Targeted delivery of TRAIL for immunotherapy of ovarian cancer

Bruyn, Marco de

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Introduction to the thesis

Introduction

A major obstacle in the treatment of cancer is the occurrence of metastasis and therapy-resistant disease relapse. A case in point here is cancer that originates from organs in the peritoneal cavity, such as ovarian carcinoma (OC). OC is the most deadly gynaecological malignancy with approximately 140,000 deaths worldwide per year [1]. Treatment of OC with curative intent typically consists of maximal surgical resection, followed by high dose chemotherapy. Unfortunately, many patients subsequently develop refractory relapses and ultimately succumb to extensive disseminated disease. These disease relapses are likely the result of several compounding factors, most notably the survival of a small subpopulation of therapy-resistant cancer cells. After a period of minimal residual disease, these therapy-resistant cells recapitulate the disease leaving few therapeutic options for intervention. Therefore, novel strategies that specifically eliminate ovarian cancer cells in disseminated and minimal residual disease are urgently needed. In this respect, the exploitation of cells and/or molecules of the human immune system is of particular interest.

In this thesis, the therapeutic concept of targeted delivery of the immune effector molecule TRAIL was evaluated in preclinical studies with an emphasis on eliminating cancer cells in disseminated and minimal residual disease

Background

Already in the early nineteen hundreds Paul Ehrlich postulated that the immune system may play a key role in the suppression of cancer. In the 1950's, this notion led Burnet and Thomas to formulate the so-called "cancer immune surveillance theory". This theory states that immune cells act as sentinels in recognizing and eliminating continuously arising (pre-) cancerous cells. Since then, numerous scientific reports have provided evidence that immune surveillance is a key defence mechanism that protects the host against the formation of tumours by maintaining normal cellular homeostasis (reviewed in [2]).

Understandably, considerable effort has therefore been spent on harnessing the immune system to treat or retard the progression of cancer, and several so-called immunotherapies have already made their way into clinical practice.

Arguably the most successful of these immunotherapeutic approaches thus far is the use of antibodies directed against cancer cell surface molecules. The beneficial effects of antibody-based therapies are largely attributable to their natural immune effector functions such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). In addition, the selectivity of antibodies has also been exploited for the targeted delivery of toxic compounds to cancerous cells. Unfortunately, authentic tumour-specific target molecules for antibody-mediated therapy are very rare. In fact, most (if not all) of the clinically used monoclonal antibodies are merely directed at overexpressed tumour-associated antigens. Therefore, conventional antibody-based approaches are often not specific

enough to allow for the delivery of highly toxic agents at clinically relevant concentrations. This concern holds particularly true for the antibody-targeted delivery of intrinsically toxic compounds, such as bacterial toxins, cytolytic drugs, and radionuclides.

Ideally, therapeutic agents used for targeted approaches are therefore inactive while “en route” and gain full anti-tumour activity only after selective binding to malignant target cells. Particularly suited in this respect is the pro-apoptotic immune ligand “Tumour Necrosis Factor (TNF)-Related Apoptosis Inducing Ligand (TRAIL)”. TRAIL is expressed as a type II transmembrane protein, but proteolytic processing or alternative splicing also yields a soluble form of TRAIL *in vivo*. This soluble form of TRAIL has been recombinantly reproduced in laboratories and has potent anti-tumour activity with no or minimal toxicity towards normal cells in numerous pre-clinical models.

TRAIL exerts its anti-tumour effects via induction of programmed cell death, also known as apoptosis. One particularly attractive feature of TRAIL is that its pro-apoptotic activity appears to be largely restricted to cells that have undergone malignant transformation. However, (pre-) clinical work has identified several important limitations that might hamper the efficacy of TRAIL-based therapies. These include the ubiquitous expression of TRAIL-receptors (TRAILRs) throughout the body and the short serum half life of TRAIL that hampers its accretion at the tumour site(s). In addition, soluble forms of TRAIL (sTRAIL) are less efficient in triggering apoptotic signalling compared to the membrane-bound form of TRAIL.

Outline of the thesis

In **chapter 2**, we highlight the rationale and perspectives of antibody fragment-based TRAIL fusion proteins for cancer immunotherapy. In this approach, sTRAIL is genetically fused to a recombinant antibody fragment directed against an overexpressed tumour-associated cell surface antigen. The resultant anti-tumour:TRAIL fusion protein has the capacity to selectively bind to the tumour cell surface and thereby convert sTRAIL into a membrane-bound form of TRAIL, with fully reactivated pro-apoptotic potential. Of particular interest in this respect is the so-called dual signalling concept. Herein, an antibody fragment with inherent anti-tumour activity is incorporated into a TRAIL fusion protein.

One prominent example of a scFv:sTRAIL fusion protein with dual signalling is the EGFR-targeted TRAIL fusion protein designated scFv425:sTRAIL. ScFv425:sTRAIL binds to EGFR as overexpressed on the cell surface of tumour cells, thereby inhibiting EGFR-mediating oncogenic signalling activity and sensitizing cells to the apoptosis induced by its sTRAIL domain. Previous studies have identified that the dual anti-tumour signalling activity of scFv425:sTRAIL resulted in potent *in vitro* anti-tumour activity towards a broad panel of carcinoma cell lines, including ovarian cancer cell lines [3].

In **chapter 3**, we expand on these promising data by evaluating the anti-tumour activity of scFv425:sTRAIL in an *in vivo* model for disseminated intraperitoneal cancer. Disseminated intraperitoneal cancers are frequently observed in advanced stage carcinomas such as OC and are a major burden for patients. After extensive disseminated tumour load had developed, mice were treated with either a high or low titre of a replication-deficient adenovirus encoding the gene for scFv425:sTRAIL (hereafter designated as Ad-scFv425:sTRAIL). High titre inoculation with Ad-scFv425:sTRAIL efficiently infected mouse livers, after which local production resulted in high serum levels of scFv425:sTRAIL. As a result, established intraperitoneal tumours were successfully eradicated. Inoculation of tumour-bearing mice with a low titre of Ad-scFv425:sTRAIL resulted in only moderate serum levels of scFv425:sTRAIL (<10 ng/mL). However, even these low serum levels of scFv425:sTRAIL were sufficient to completely halt further tumour growth. Together, these data highlight the potential of scFv425:sTRAIL for the treatment of intraperitoneal disseminated cancer.

In **chapter 4**, the scFv425:sTRAIL concept was modified by using a rationally-designed variant of sTRAIL with engineered enhanced binding affinity for TRAILR1. We speculated that the use of such a sTRAIL variant would further enhance the induction of apoptosis in cancer cells expressing TRAILR1 over TRAILR2. This novel fusion protein, designated scFv425:sTRAILmR1-5, showed superior anti-cancer activity in a broad panel of cancer cell lines, irrespective of the relative expression of TRAILR1 over TRAILR2. Moreover, the synergistic anti-tumour activity of scFv425:sTRAILmR1-5 was superior to scFv425:sTRAIL-wt, when treatment was combined with cytostatic drugs. These data indicate that the use of rationally-designed sTRAIL mutants in the scFv:sTRAIL fusion protein concept results in an improved therapeutic activity.

Cancer patients that are diagnosed early with local cancer lesions usually have good prospects for curative treatment. In contrast, patients diagnosed with metastatic disease will, even after seemingly complete tumour eradication, ultimately succumb due to occult metastatic and refractory disease. A particularly important factor is the potential presence of circulating cancer cells that, after period of minimal residual disease, ultimately initiate formation of distant metastasis. Prevention and/or inhibition of metastases formation by circulating or occult cancer cells is therefore paramount to achieving curative treatment. Therefore, we hypothesized that a rationally-designed multi-functional scFv:sTRAIL fusion protein that inhibits metastatic spread of circulating tumour cells while simultaneously inducing tumour cell-restricted apoptosis may have considerably enhanced therapeutic activity. To this end we used a scFv targeting domain that has intrinsic anti-metastatic potential in its own right. In **chapter 5**, this approach was evaluated in a model system for malignant melanoma, a disease characterized by a rapid metastatic spread. To this end,

a novel anti-melanoma scFv:sTRAIL fusion protein, designated anti-MCSP:TRAIL, was constructed using a scFv antibody fragment derived from mAb 9.2.27. MAb 9.2.27 is directed against the Melanoma Chondroitin Sulphate Proteoglycan (MCSP), an established melanoma-associated target antigen that is highly expressed on both primary tumours and metastatic lesions. Furthermore, previous reports indicated that MSCP signalling is implicated in the metastatic potential of melanoma cells. Anti-MCSP:TRAIL potently induced apoptosis in a series of melanoma cell lines and inhibited outgrowth of melanomas both *in vitro* and *in vivo*. Interestingly, TRAIL-resistant melanoma cells were also effectively inhibited in their outgrowth. We speculated that the TRAIL-independent growth inhibition observed for anti-MSCP:sTRAIL might be largely attributable to its inhibitory activity towards MCSP signalling. Previous reports indicated that treatment with the parental anti-MCSP monoclonal antibody mAb 9.2.27 resulted in the inhibition of pro-metastatic signalling pathways. Indeed, using kinase arrays, we demonstrated that treatment with anti-MCSP:TRAIL inhibited several kinases implicated in melanoma cell growth and metastasis. We conclude that fusion protein anti-MCSP:TRAIL has promising pre-clinical anti-melanoma activity that appears to result from combined inhibition of tumorigenic MCSP-signalling and concordant activation of TRAIL apoptotic signalling.

The work presented in chapters 2 to 5 focuses on the direct delivery of TRAIL to the cell surface of tumour cells and establishes that the scFv:sTRAIL concept: 1. has *in vivo* activity towards solid cancers, 2. can be optimised by incorporating rationally-designed TRAIL mutants, 3. is effective at inhibiting metastatic features of cancer cells, and 4. has no or minimal activity towards normal cells.

In **chapter 6**, we evaluated a different approach in which TRAIL is targeted to the cell surface of immune effector cells in an attempt to augment their TRAIL-mediated tumoricidal activity. As already detailed in chapter 2, TRAIL is an immune effector molecule that is expressed on the cell surface of natural killer (NK) cells, where it is essential for NK cell–mediated tumour immune surveillance. In contrast, the expression of TRAIL on resting T lymphocytes (or T-cells) is typically very low. However, already a slight upregulation of TRAIL on the cell surface of T cells, as induced by e.g. *ex vivo* co-stimulation with anti-CD3 antibodies and IFN-α, significantly increases their tumoricidal activity. This suggests that T-cells may substantially gain in tumoricidal potential when additional TRAIL is selectively delivered to their cell surface. This hypothesis was investigated by selectively delivering TRAIL to the T-cell surface molecules CD3 and CD7. To this end, we constructed two novel TRAIL fusion proteins, designated anti-CD3:TRAIL and K12:TRAIL, harbouring an anti-CD3 scFv and the CD7-ligand K12 respectively. Incubation of T-cells with these fusion proteins resulted in an up to 500-fold increase of their *in vitro* tumoricidal activity towards a panel of cancer cell lines and towards primary patient-derived ovarian cancer

cells. Importantly, both anti-CD3:TRAIL and K12:TRAIL potently inhibited the outgrowth of disseminated intraperitoneal carcinoma xenografts *in vivo*. Together, these preclinical data indicate that targeted delivery of TRAIL to the cell surface of T-cells potently enhances their anti-tumour activity. Of note, this novel concept can easily be incorporated into current clinically applied T-cell-based immunotherapeutic strategies.

As described above, TRAIL can be used to augment the anti-tumour activity of immune effector cells. Interestingly, TRAIL treatment may also have other immune stimulating effects. In this respect, it has been reported that treatment with certain pro-apoptotic agents, including TRAIL, triggers translocation of the ER-resident chaperone protein calreticulin (CRT) to the tumour cell surface. Here, CRT appears to serve as an 'eat me' signal for phagocytes. It is well-established that the uptake of pre-apoptotic cells by conventional phagocytes is largely non-immunogenic. However, recently it was uncovered that CRT-exposing pre-apoptotic cells are preferentially phagocytosed by dendritic cells, which may lead to the induction of an antitumor immune response.
Recent *in vitro* findings, obtained by using an ELISA-type binding assay, indicated that CRT can directly interact with TRAIL. Therefore, in **chapter 7**, we evaluated the role of CRT in TRAIL-mediated cell death. We demonstrated that treatment of tumour cells with TRAIL induced the rapid pre-apoptotic association of CRT with TRAIL and TRAILR2. Concurrently, CRT dissociated from its normal binding partner CD47. Of note, dissociation of CD47 from CRT has been reported to enhance clearance of apoptotic cells by phagocytes. Interestingly, the association of TRAIL with CRT had no effect on the induction of apoptosis. In conclusion, association of TRAIL with CRT appears to alter cell surface distribution of CRT (and other immune molecules) and as such might have immunological implications for e.g. phagocytosis and immunogenicity of apoptotic tumour cells.

A summary of the work performed in this thesis is provided in **chapter 8** along with perspectives on the further development of targeted delivery of TRAIL for optimizing immunotherapy.

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Targeted cancer immunotherapy using ligands of the Tumor Necrosis Factor super-family

Marco de Bruyn[1], Edwin Bremer[1], Harald Wajant[2] and Wijnand Helfrich[1]

[1]Department of Surgery, Surgical Research Laboratories, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

[2]Division of Molecular Internal Medicine, Department of Internal Medicine II, University Hospital Würzburg, Germany.

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Abstract

Ideally, an immunotoxin should be inactive 'en route', acquire activity only after tumor cell surface binding and have no off-target effects towards normal cells. In this respect, antibody-based fusion proteins that exploit the tumor-selective pro-apoptotic death ligands sFasL and sTRAIL appear promising. Soluble FasL largely lacks receptor activating potential, whereas sTRAIL is inactive towards normal cells. Fusion proteins in which an anti-tumor antibody fragment (scFv) is fused to sFasL or sTRAIL prove to be essentially inactive when soluble, while gaining potent anti-tumor activity after selective binding to a predefined tumor-associated cell surface antigen. Importantly, off-target binding by scFv:sTRAIL to normal cells showed no signs of toxicity. In this review, we highlight the rationale and perspectives of scFv:sTRAIL/ scFv:sFasL based fusion proteins for cancer therapy.

Introduction

Since the advent of targeted cancer therapy decades ago significant progress has been made in the development of drugs that display enhanced tumor-selective activity. Antibody-targeted agents in particular have demonstrated potent activity both in pre-clinical and clinical studies and have had a positive effect on treatment outcome in several human malignancies [1]. In many cases, antibodies or fragments thereof are equipped with potent toxic effector moieties of bacterial or plant origin. An example worth mentioning is the immunotoxin Mylotarg, which comprises an anti-CD33 antibody chemically linked to the plant toxin calicheamicin. Mylotarg has been approved for a subgroup of Acute Myeloid Leukemia patients and has yielded promising results [2]. Nevertheless, many forms of targeted therapeutics are hampered by dose-limiting toxicity towards healthy cells. Such toxicity can be due to a lack of tumor specificity of the antibody and/or a lack of tumor-selective activity of the cytotoxic effector molecule employed.

An attractive strategy to reduce toxicity towards normal cells and optimize therapeutic efficacy of targeted therapy is to harness the body's own effector mechanisms for the elimination of tumor cells. The immune system is equipped with potent tumoricidal molecules that may be exploited to effectively eliminate malignant cells with limited to no activity towards non-transformed cells. In this respect, members of the tumor necrosis factor (TNF) family and more specifically the subgroup of Death Inducing Ligands are of particular interest [3]. Of this small subgroup, the Fas ligand (FasL) and TNF-related Apoptosis Inducing Ligand (TRAIL) appear well suited for integration in antibody-targeted cancer therapy. As immune effector molecules, FasL and TRAIL are both primarily involved in the induction of programmed cellular death, better known as apoptosis, in virus infected and in malignant cells [4].

On immune effector cells, FasL and TRAIL are expressed as type II trimeric transmem-

brane proteins. However, *in vivo* proteolytic processing or alternative splicing can also yield soluble forms of these death ligands [5]. These soluble ligands have a different activity profile than the corresponding membrane-bound form. The soluble form of FasL (sFasL) almost entirely lacks receptor activating potential for its agonistic receptor Fas [6-8]. The soluble form of TRAIL (sTRAIL) on the other hand retains receptor-activating potential for its agonistic receptor TRAIL-R1, but fails to efficiently activate the other agonistic receptor, TRAIL-R2 [9-12]. Intriguingly, sTRAIL has been found to specifically lack toxicity towards normal cells, whereas sFasL is largely inactive per se. An important finding that highlights the potential for use of these ligands for antibody-targeted therapy is the reacquisition of full membrane-like activity upon artificial oligomerization of sTRAIL and sFasL [13;14]. Previously, we and others reported on a novel class of anti-cancer agents in which a tumor-selective single chain fragment of variable regions (scFv) antibody fragment is genetically fused to soluble sTRAIL and/or sFasL, yielding scFv:sTRAIL and scFv:sFasL fusion proteins, respectively. Upon tumor-selective binding, these largely inactive fusion proteins are locally converted to membrane-bound molecules with fully restored pro-apoptotic anti-tumor activity.

In this review, we will first briefly detail the basics of FasL and TRAIL–mediated apoptotic signaling events. Next, we will discuss the current status of non-targeted FasL and TRAIL in cancer therapy. Subsequently, we will highlight advances in the development of targeted FasL- and TRAIL-based therapeutic strategies to achieve optimal anti-tumor potential with minimal toxicity towards normal cells.

Apoptotic signaling

FasL and TRAIL eliminate cells by apoptosis, a pivotal homeostasis mechanism by which superfluous, damaged, or dangerously altered cells are removed in a non-immunogenic manner [4]. At the heart of apoptotic signaling is the coordinated activation of a group of cellular cysteine proteases – the effector caspases – that cleave a variety of cellular substrates. These activated effector caspases ultimately induce apoptotic cell death. Activation of effector caspases is carefully orchestrated by a subgroup of caspases known as initiator caspases. All caspases are produced as pro-enzymes that require proteolytic processing to unleash their catalytic activity. The initiator caspases can be activated via two main routes designated the Death Receptor (or extrinsic) pathway and the mitochondrial (or intrinsic) pathway (see Fig. 1 for overview). The Death Receptor pathway centers on the activation of cell surface expressed transmembrane Death Receptors (DR) by Death Inducing Ligands, such as FasL and TRAIL. Binding of TRAIL to its cognate agonistic receptor triggers the formation of a pro-apoptotic protein complex termed the Death Inducing Signaling Complex (DISC) at the cytoplasmic death domain (DD) of the Death Receptors [15]. The adaptor protein Fas-associated death domain (FADD), is subsequently recruited

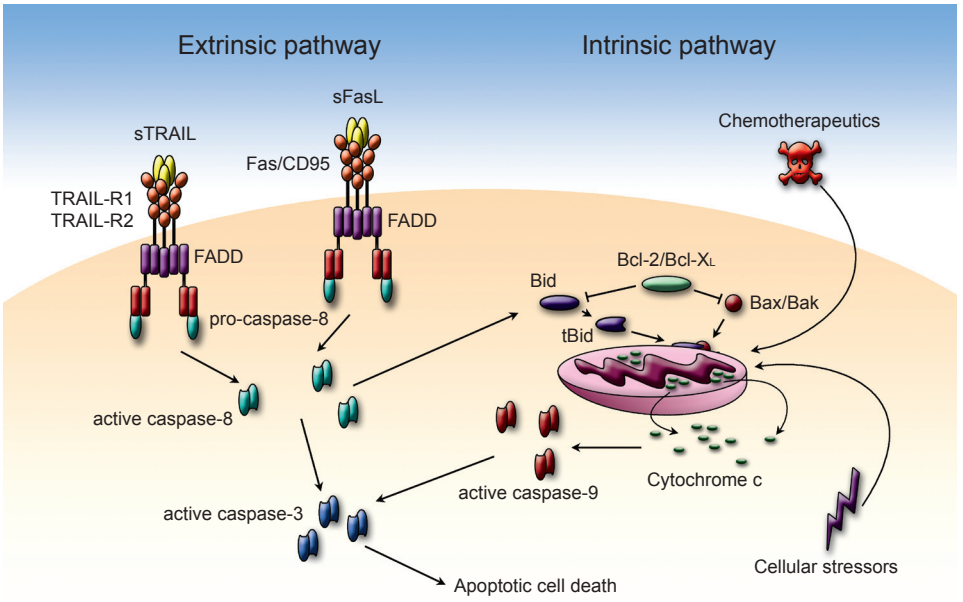

Figure 1. Molecular mechanisms of apoptosis. The extrinsic pathway of apoptosis is activated upon ligation of TRAIL or FasL to cell surface expressed transmembrane receptors TRAIL-R1 / TRAIL-R2 and Fas, respectively. Binding of these death inducing ligands to their cognate receptors triggers the formation of a pro-apoptotic protein complex termed the Death Inducing Signaling Complex (DISC) at the cytoplasmic death domain (DD) of TRAIL-R1, TRAIL-R2 or Fas. A DD-containing protein, dubbed Fas-associated death domain (FADD), is subsequently recruited together with pro-caspase-8. In the DISC, initiator pro-caspase-8 is proteolytically activated, whereupon caspase-3 is proteolytically activated, leading to execution of apoptosis. The intrinsic pathway of apoptosis is activated upon signals of cellular stress or after stimulation with most apoptosis inducing chemotherapeutics. The hallmark of this intrinsic pathway is mitochondrial membrane depolarization, leading to the subsequent release of cytochrome C and activation of initiator caspase-9. Mitochondrial apoptosis is controlled by the Bcl-2 family, which consists of both pro- (Bid, Bax and Bak) and anti- (Bcl-2 and Bcl-X_L) apoptotic proteins. In addition, a mitochondrial amplification loop can be activated by caspase-8-mediated cleavage of the BH3-only interacting domain death agonist (BID). Cleavage of BID into truncated BID (tBID) results in its mitochondrial translocation, where it associates with Bax and Bak and triggers mitochondrial depolarization.

to the DD of the receptor together with pro-caspase-8. In the DISC, initiator caspase-8 is proteolytically activated, whereupon the proteolytic caspase cascade is triggered, leading to execution of apoptosis [16].

The hallmark of the mitochondrial pathway of apoptosis is the depolarization of the mitochondrial membrane, leading to release of cytochrome C and subsequent activation of

initiator caspase-9. Caspase-9 in turn activates executioner caspase-3. The mitochondrial depolarization is governed by the Bcl-2 family, a family of proteins consisting of both pro- and anti-apoptotic proteins [17]. Most cellular stressors, including conventional chemotherapeutics and radiation, induce apoptosis by shifting the balance towards the pro-apoptotic Bcl-2 family members, thereby triggering execution of the mitochondrial pathway of apoptosis.

Crosstalk between the extrinsic and intrinsic apoptotic signaling pathways exist, as signaling induced via Death Receptors can be amplified through a so-called mitochondrial amplification loop. This loop is activated by caspase-8 mediated cleavage of the BH3-only interacting domain death agonist (BID). Truncated BID (tBID) can then trigger mitochondrial depolarization and activation of caspase-9.

FasL and TRAIL biology

FasL signals apoptosis by binding to the trimeric receptor Fas (also known as CD95), after which Fas trimers reorganize into higher order molecular structures [18;19]. These receptor clusters are focal points for the formation of the above described DISC and form the starting point for apoptotic signaling by Fas. In addition, FasL can also bind to Decoy Receptor 3 (DcR3) [20]. DcR3 is a soluble receptor that lacks an intracellular DD and cannot recruit adaptor proteins required for apoptosis. Therefore, the main function of DcR3 appears to be inhibition of FasL-apoptotic activity [21]. Indeed, high circulating levels of DcR3 protect synovial fibroblasts against FasL signaling in rheumatoid arthritis [22].

TRAIL interacts with an elaborate receptor system comprising two agonistic receptors, TRAIL-R1 and TRAIL-R2, two antagonistic receptors, TRAIL-R3 and TRAIL-R4, and the soluble receptor osteoprotegrin (OPG) [23]. Both agonistic receptors TRAIL-R1 and TRAIL-R2 contain a functional intracellular DD and can induce apoptotic signaling via DISC formation. The antagonistic receptor TRAIL-R3 is glycosyl-phosphotidylinositol (GPI)-linked and lacks an intracellular domain, whereas TRAIL-R4 only has a truncated and non-functional DD. These two receptors are, analogous to DcR3 for FasL, thought to function as decoy receptors that modulate TRAIL-sensitivity although the precise mechanism has yet to be characterized. Recent work suggests that TRAIL-R3 can bind and sequester TRAIL in lipid membrane microdomains [24]. TRAIL-R4 on the other hand appears to form heterotrimers with TRAIL-R2 and thereby disrupts TRAIL-R2 mediated apoptotic signaling [24;25]. Of note, the mechanism behind the resistance of normal cells to TRAIL remains unclear and is a subject of intense interest and debate for many researchers. It has been speculated that overexpression of antagonistic TRAIL-receptors, differential formation of heterotrimeric and inactive TRAIL-receptors, as well as cancer-specific post-translational modifications may be involved in the tumor-selective activity of TRAIL. The recently described correlation between O-glycosylation of TRAIL-receptors and apoptosis induction following TRAIL-

ligation may provide further clues [26]. As we will discuss further on, various aspects of this complex ligand/receptor system may be exploited to optimize the efficacy of TRAIL-based therapy.

Non-targeted sFasL for cancer therapy

Early studies with only partially characterized sFasL preparations and agonistic Fas antibodies demonstrated potent apoptotic activity towards a host of tumor cell lines and primary human tumor cells [18]. In principle, this feature qualified FasL as an interesting candidate for cancer therapy. Unfortunately, systemic administration of these Fas agonists was associated with severe liver toxicity in mice [27]. However, it is now well established that homogenous trimeric sFasL preparations that do not contain higher order molecular aggregates are largely inactive and in fact competitively inhibit the pro-apoptotic activity of membrane-expressed FasL [6;7]. Of note, secondary cross-linking of trimeric sFasL to a hexameric state is already sufficient to largely restore its pro-apoptotic activity [13;14]. Of considerable interest in this respect is an engineered hexameric FasL variant, dubbed MegaFasL (MFL). MFL induced apoptosis in cell lines and primary cells from multiple myeloma (MM), acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and Burkitt's lymphoma [14]. Furthermore, induction of apoptosis by MFL in gastro intestinal tumors (GIST) was potentiated by co-treatment with the Bcr-Abl tyrosine kinase inhibitor imatinib [28]. Importantly, MFL did not induce apoptosis in normal hematopoietic progenitor cells [14]. Moreover, i.p. administration of therapeutic doses of MFL in a xenograft model of ovarian carcinoma resulted in only mild and transient signs of liver toxicity. Histological analysis performed after cessation of therapy revealed that the liver damage was of reversible nature indicating that prudent development for clinical applications appears warranted [29].

Although highly active against a wide variety of tumor cell lines, resistance to FasL has been reported in various tumor types, e.g. prostate cancer. Specifically, prostate cancer cells with an androgen-independent phenotype appear to upregulate anti-apoptotic molecules like $cFLIP_L$ [30]. Therefore, combinatorial strategies that sensitize cells to the pro-apoptotic activity of FasL are of particular interest. Interestingly, the inhibition of AMP-activated protein kinase (AMPK) was found to enhance cFLIP degradation in the proteasome thereby sensitizing prostate cancer cells to FasL-induced apoptosis [31]. In addition, many conventional chemotherapeutic agents like mitoxantrone and docetaxel potently enhanced FasL induced apoptosis in both cell lines and primary prostate cancer samples by, among others, increasing expression of Fas [32].

Targeted delivery of FasL for cancer therapy

As described above, a homogenous trimeric sFasL preparation is devoid of pro-apoptotic activity, but can be readily reactivated by secondary crosslinking. Therefore, sFasL is argu-

ably a promising candidate as effector molecule in antibody-targeted approaches. Antibody-targeted sFasL, such as in our scFv:sFasL fusion protein format, remains inactive while en route but is converted to its fully active membrane-like form upon antibody-mediated binding to the tumor cell surface. The feasibility of this concept has been demonstrated by us and others. In a first report, targeted delivery of sFasL to the tumor stroma marker FAP, using an anti-FAP scFv resulted in a 1000-fold increase in specific activity to FAP-positive cells compared to untargeted sFasL [33] (see Table 1 for overview of reported scFv:sFasL fusion proteins). Importantly, this fusion protein remained inactive towards FAP-negative cells [33]. Similarly, targeted delivery of sFasL to the T-cell marker CD7, using the fusion protein scFvCD7:sFasL, induced potent CD7-restricted apoptosis in T-cell leukemic cell lines and primary patient-derived leukemic cells. In contrast, CD7-positive resting lymphocytes were fully resistant to prolonged treatment with the fusion protein scFvCD7:sFasL [34].

Of note, during the resolution phase of an immune response, activated T-cells are effectively eliminated via a process called activation-induced cell death, a process in which FasL is a main pro-apoptotic cell surface molecule. In accordance with this physiological role, *ex vivo* activated T-cells were found to be highly susceptible to scFvCD7:sFasL mediated induction of apoptosis. This feature might be of therapeutic interest in elimination of activated autoreactive T-cells in inflammatory diseases such as rheumatoid arthritis [35].

A promising strategy in the design of scFv:sFasL fusion proteins is the incorporation of a scFv fragment with intrinsic pro-apoptotic activity. The promise of this approach is illustrated by a recently generated fusion protein comprising a rituximab-derived antibody fragment (scFvRit) and sFasL [36]. Rituximab binds to the B-cell antigen CD20 and is already in clinical practice with considerable success. In patients, rituximab exerts its effect partly through induction of apoptosis by crosslinking of the CD20 target molecule [37]. Interestingly, we and others have previously identified that co-treatment of B-cell leukemic cell lines with rituximab and Fas-agonistic antibodies yielded a synergistic induction of apoptosis [38;39]. The CD20-directed fusion protein scFvRit:sFasL further capitalized on this co-treatment approach which is evidenced by the superior levels of apoptosis this fusion protein induced in CD20-positive malignant cells compared to co-treatment with rituximab and FasL. In contrast, CD20-negative cells were not affected by scFvRit:sFasL. This superior apoptotic activity of scFvRit:sFasL suggests a unique cooperative effect of simultaneous CD20 crosslinking and Fas-activation. Considering the documented requirement for crosslinking of both CD20 and Fas to achieve activation, it is conceivable that scFvRit:sFasL induces mutual spatial rearrangements in CD20 and Fas distribution, thereby enhancing pro-apoptotic signaling events.

Non-targeted TRAIL for cancer therapy

TRAIL is probably one of the more promising anti-tumor agents. This promising status is based on the pronounced selective activity of TRAIL towards a variety of malignant cells and its lack of activity towards normal cells. Treatment with TRAIL induced apoptosis in over 40% of all tumor cell lines tested, including tumors of hematopoietic origin as well

Table 1. scFv:sTRAIL and scFv:sFasL fusion proteins reported

Fusion protein	Target antigen	Target tumor	*In vivo*	Combination with other anti-cancer therapeutics
MBOS4-TRAIL[10]	Fibroblast activation protein (FAP)	Tumor stroma	No	-
scFvC54:sTRAIL[59;60]	Epithelial cell adhesion molecule (EpCAM)	Carcinoma	Yes*	-
scFv425:sTRAIL[61]	Epidermal Growth Factor Receptor (EGFR)	Glioblastoma, Ovarian cancer, Colon cancer, Lung cancer	Yes[58]	Iressa[61], Valproic acid[50], Cisplatin[50]
scFv425:sTRAIL-mR1-5[50]	Epidermal Growth Factor Receptor (EGFR)	Glioblastoma, Ovarian cancer, Colon cancer, Lung cancer	No	Valproic acid[50], Cisplatin[50]
scFvCD7:sTRAIL[56]	CD7	T-cell leukemia	No	UCN01[56], Vincristin[56], Cyclohexamide[56]
scFvCD19:sTRAIL[57]	CD19	B-cell leukemia	Yes[57]	Valproic acid[57]
scFvCD33:sTRAIL[2]	CD33	Acute myeloid leukemia (AML)	No	Mitoxantrone[2], Vaplroic acid[2], 17-AAG[2], Gleevec[2]
sc40-FasL[33]	Fibroblast activation protein (FAP)	Tumor stroma	Yes[33]	-
scFvCD7:sFasL[34]	CD7	T-cell leukemia	No	Vincristin[34], L-744,832[34], Velcade[34]
scFvRit:sFasL[36]	CD20	B-cell leukemia	Yes[36]	-
SC36-CD95L-PD[75] (and related pro-drug constructs)	Fibroblast activation protein (FAP)	Tumor stroma	Yes[75]	-

as solid tumors [40]. In addition, TRAIL showed marked anti-tumor activity in xenografted tumor models in mice [40]. By now sTRAIL has advanced to phase 1 clinical testing. Preliminary reports from these trials indicate promising results in terms of tolerability of sTRAIL in humans, with no major adverse events documented [41-46]. Particularly promising is the fact that in a small clinical trial with combination treatment of B-CLL patients with sTRAIL

and rituximab 2 complete responses and 1 partial response were reported in a group of 4 patients [40;44]. In addition, a recent and larger phase I clinical trial of recombinant human rhTRAIL/APO2L in 71 patients with advanced cancer reported no major adverse effects and stable disease or better in 46% of patients after 6 months [46].

The aforementioned complexity of TRAIL-receptor signaling may further offer ways of designing tumor-tailored strategies. For instance, evidence indicates that apoptotic signaling in certain tumors, such as chronic myeloid leukemia and pancreatic cancer is mainly mediated via TRAIL-R1, while other tumor types appear to be primarily susceptible to TRAIL-R2 apoptotic signaling [11;47-49]. This differential use of agonistic TRAIL receptors may be exploited by the use of TRAIL-receptor selective sTRAIL mutants with enhanced specificity for the most prevalently expressed TRAIL-receptor in a given tumor type. In addition, decreasing the affinity of sTRAIL for decoy receptors may offer significant advantages in terms of reducing retention in normal tissue and optimizing occupancy of agonistic TRAIL-receptors. To date, several sTRAIL mutants with increased selectivity for one of the agonistic receptors have been reported in preclinical studies [11;48-50]. It will be interesting to see whether one of these mutants may prove more effective towards pre-selected tumor types. Obviously, assessment of potential toxicity due to the introduced amino acid modifications will need to be carefully assessed. In addition, these modifications may also yield new immunogenic epitopes, whereby clinical applicability could be hampered due to the development of anti-TRAIL antibody responses.

Despite the obvious and considerable promise of TRAIL-based therapy its clinical efficacy may be hampered by the intrinsic resistance to TRAIL observed in various tumor types. This is exemplified by primary patient-derived glioma and hepatoma cells of which both display an almost complete resistance to TRAIL-induced apoptosis [51]. Importantly, TRAIL-signaling synergizes with most commonly used cytostatic agents and also with novel therapeutics. The combinatorial synergistic activity of TRAIL with conventional anticancer drugs is reflected by a host of publications on preclinical studies both *in vitro* and *in vivo* [52-55]. Indeed, co-treatment regimes appear the most promising way forward to overcome TRAIL resistance in human malignancies, with various points of crosstalk between the extrinsic and the intrinsic apoptotic pathway as possible therapeutic targets.

Targeted delivery of sTRAIL for cancer therapy

In addition to possible resistance issues, other characteristics of the TRAIL/TRAIL receptor system may hamper the clinical efficacy of sTRAIL. Firstly, sTRAIL has a serum half-life of only ~30 minutes in humans, which is likely to severely limit tumor accretion [11;41]. Secondly, TRAIL-receptors are ubiquitously expressed throughout the body, possibly acting as a sink for sTRAIL that may preclude efficient tumor accretion. Lastly, TRAIL-R2 signaling is relatively poorly activated by sTRAIL, whereas many tumor types frequently express

TRAIL-R2 over TRAIL-R1 [9].

Most of these potential limitations of sTRAIL may be overcome by fusion of a tumor-specific scFv to sTRAIL. The resultant scFv:sTRAIL fusion protein is homotrimeric with a molecular weight of approximately 150 kDa. As a consequence, glomerular excretion of scFv:sTRAIL should be decreased, thereby increasing the retention time in the circulation. Furthermore, accretion of scFv:sTRAIL at the tumor cell surface should be significantly increased by virtue of its high binding affinity for the cell surface target antigen of choice [50;56-58]. Of note, since scFv:sTRAIL fusion proteins are trimers, three scFv reading heads are available yielding a strongly enhanced binding avidity. Importantly, we and others have shown that scFv:sTRAIL is capable of efficiently activating not only TRAIL-R1 but also TRAIL-R2 [10;59]. Upon targeted delivery of sTRAIL to the pan-carcinoma marker EpCAM, membrane-bound scFv:sTRAIL induces apoptosis in a mono- and/or bi/multi-cellular manner (Fig. 2) [60]. This bi/multi-cellular mode of action enables the elimination of tumor cells devoid of target antigen by neighboring target antigen-positive and scFv:sTRAIL loaded cells, a feature termed the bystander effect [56;60]. This may be particularly relevant for tumors known to be heterogeneous for tumor-associated antigens, such as glioblastoma multiforma. Proof of concept for several scFv:sTRAIL fusion proteins has been obtained including those with specificity for the tumor stroma antigen FAP, solid tumor antigens EpCAM and EGFR as well as various leukemia target antigens (CD7, CD19, CD33) [2;10;50;56;57;61] (see Table 1 for overview).

For our studies we have had a particular interest in scFv fragments with intrinsic anti-tumor activity. The anti-tumor activity of an scFv may come from direct pro-apoptotic activity, or by its ability to competitively inhibit survival signaling, thereby sensitizing targeted cells to TRAIL-mediated apoptosis. The dual activity of such a bifunctional scFv:sTRAIL fusion protein is exemplified by fusion protein scFv425:sTRAIL, which contains the EGFR blocking scFv425 [61]. Binding of scFv425:sTRAIL to EGFR-positive carcinoma cells inhibited mitogenic signaling and its subsequent downstream pro-survival signaling events. EGFR signaling inhibition by scFv425:sTRAIL resulted in, among others, down-regulation of cFLIP. Concordantly, the TRAIL domain activated TRAIL-R apoptotic signaling. Both *in vitro* experiments using various EGFR-positive cell lines and *in vivo* experiments in $EGFR^+$ tumor bearing mice demonstrated highly potent anti-tumor activity for scFv425:sTRAIL [58;61]. This strategy is probably applicable to a host of other growth factor receptors, such as insulin growth factor receptor (IGFR) and human epidermal growth factor receptor 2 (HER2). The promise of combinatorial Growth Factor Receptor targeting and TRAIL-R activation is further highlighted by a report in which mAbs directed at HER2 and TRAIL-R2 yielded synergistic elimination of tumor cells both *in vitro* and *in vivo* [62].

It is worth noting here that pro-survival signaling has also recently been reported for the pan-carcinoma associated target antigen EpCAM [63]. Upon cell-cell contact, the intracel-

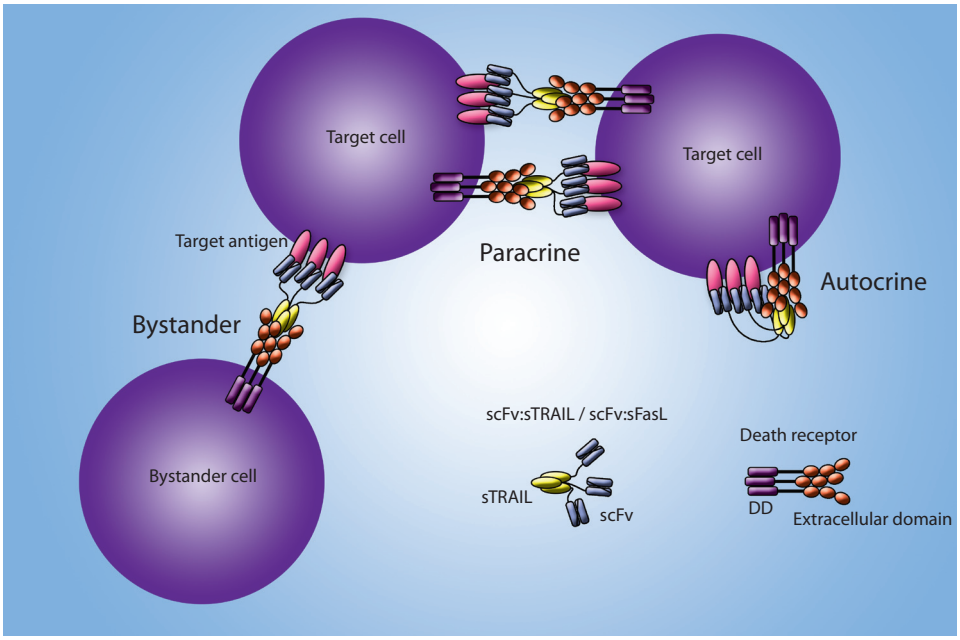

Figure 2. Target-cell-restricted induction of apoptosis by scFv:sTRAIL and scFv:sFasL fusion proteins. Specific binding of scFv:sTRAIL and scFv:sFasL to the target antigen results in accretion at the tumor cell surface. Subsequently apoptosis can be induced in an autocrine manner by binding to the cognate death receptors on the same target tumor cell. Similarly, specific binding of scFv:sTRAIL and scFv:sFasL fusion proteins to antigen on one target tumor cell can induce activation of cognate death receptors on a neighboring antigen-expressing target tumor cell resulting in a paracrine target cell apoptosis. In addition, target antigen-positive and scFv:sFasL or scFv:sTRAIL loaded cells can present sFasL or sTRAIL to neighboring cells devoid of antigen and thereby enable elimination of these bystander tumor cells in a feature termed the bystander effect.

lular region of EpCAM, dubbed EpCAM IntraCellular Domain (EPICD), was found to be cleaved from the membrane upon which it translocated to a transcription factor complex in the nucleus. In the nucleus, EPICD activated among others the proto-oncogene c-Myc leading to pro-mitogenic signaling. In light of these new data, it is tempting to speculate that the potent apoptotic activity we found for the EpCAM-specific fusion protein scFvC54:sTRAIL may be partly related to inhibition of EpCAM processing and concomitant inhibition of mitogenic cell signaling pathways.

The activity of antibody-targeted TRAIL might be further optimized by incorporating the aforementioned TRAIL-receptor selective mutants into the fusion protein concept. Indeed, a variant of fusion protein scFv425:sTRAIL, containing a TRAIL mutant with increased activity towards TRAIL-R1, demonstrated significantly higher apoptotic activity in roughly 50%

of all cell lines tested (ref [50]; see also chapter 4).

Conclusions and perspectives

As discussed in this review, TRAIL as well as FasL are immune effector molecules that may be of considerable interest for antibody-targeted cancer therapy. Genetic fusion of a tumor-selective antibody fragment to sTRAIL and sFasL yields highly selective anti-cancer therapeutics with favorable anti-cancer features. Antibody-based targeting helps to exploit several of the intrinsic characteristics of the interaction of these ligands with their cognate receptors to ensure minimal off-target activity and enhanced tumoricidal activity. Important features of this approach include the selective accretion to the tumor cell surface and subsequent activation of sTRAIL and sFasL to full membrane-like anti-tumor activity. The use of antibody fragments in such fusion proteins that have intrinsic tumoricidal activity further provides possibilities to optimize tumoricidal activity by simultaneously exploiting key signaling events in cancer cells. Such a rationally designed approach may significantly synergize the therapeutic potential of antibody-based death ligand fusion proteins.

The most likely application of sTRAIL and sFasL based antibody fusion proteins is in the management of residual disease and prevention of cancer relapse. Of specific interest in this respect is the fact that cancer relapse in many types of tumors is currently being attributed to so-called cancer stem cells (CSCs). These CSCs represent a small subpopulation of cancer cells that display a stem cell-like phenotype and are particularly resistant to conventional cancer therapy. Therefore, CSCs are held responsible for disease relapse and their targeted elimination might prove crucial for potentially curative treatment. Interestingly, TRAIL significantly inhibited the clonogenic capacity of acute myeloid leukemia ($CD34^+$) CSCs, without affecting normal $CD34^+$ cells [64]. Similarly, colon cancer cells with CSC-properties were found to be more sensitive to TRAIL-induced apoptosis [65], although others have found that primary colorectal CSCs were relatively resistant to TRAIL [66]. However, this TRAIL-resistant phenotype can be fully reverted by blocking of autocrine pro-survival IL4 signaling [66]. These findings provide a clear incentive for further investigation of the therapeutic activity of sTRAIL and antibody-targeted TRAIL for the elimination of cancer stem cell populations. An attractive option in the latter case may be the incorporation of a blocking IL4R antibody. In addition, since carcinoma CSCs are usually identified by the expression of high levels of EpCAM [67], we are currently re-evaluating the activity of our previously reported EpCAM-targeted scFvC54:sTRAIL fusion protein towards the CSCs subpopulation.

A potential limitation to the scFv:sFasL/scFv:sTRAIL fusion protein approach discussed in this review is the potential limitation in tumor-penetration in the case of solid tumors. Antibody fragment targeted death ligands are ~twice the size of non-targeted soluble ligands and thus will be more limited in diffusing through multiple cellular layers. Indeed, the most

likely application of scFv:sFasL/scFv:sTRAIL in solid tumors is in conjunction with surgery and/or chemotherapeutics, i.e. to eliminate minimal residual disease. In addition, tumor-endothelial targeted approaches analogous to those reported for TNF are of interest. In such cases, the tumor endothelium will be damaged and the surrounding tumor cells will be devoid of crucial supply of oxygen. An added benefit of this approach, as also evident from the clinical experience with TNF, would be the enhanced diffusion of any co-administered chemotherapeutics or for that matter cancer cell surface-targeted scFv:sTRAIL and scFv:sFasL or combinations thereof.

Finally, as more of the specific molecular aberrations responsible for malignant behavior of cancer cells are identified, the design of rational combinatorial strategies will take an increasingly prominent place in targeted cancer therapy. Preclinical data provide clear indications of the promise of combinatorial strategies directed at modulating apoptotic Death Receptor signaling by conventional or experimental therapeutics [68]. Of note, although Death Receptors predominantly signal apoptosis, oncogenic proteins like K-Ras and NF-κB can potentially turn death receptors into metastasis-promoting receptors [69-72]. Converting metastatic death-receptor signaling to apoptotic signaling via K-Ras or NF-κB inhibition might therefore provide further therapeutic benefit. An important factor to be kept in mind during design of any combinatorial strategy is the potential for unwanted side-effects. In particular, hepatocytes appear to be one of the most vulnerable populations that need to be carefully monitored [73]. For instance, co-treatment of TRAIL with proteasome inhibition also resulted in liver toxicity, albeit within a therapeutic window [74].

In conclusion, scFv-targeted FasL and TRAIL fusion proteins have yielded promising preclinical data that warrants further clinical evaluation. It will be exciting to see whether these novel therapeutics can indeed fulfill the promise of enhanced cancer selective activity and minimal side effects.

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Potent systemic anti-cancer activity of adenovirally expressed EGFR-selective TRAIL fusion protein

Edwin Bremer[1,2*], Gooitzen M. van Dam[3,4*], Marco de Bruyn[1], Manon van Riezen[1], Marike Dijkstra[5], Gera Kamps[5], Wijnand Helfrich[1±], Hidde Haisma[5±]

[1]Groningen University Institute for Drug Exploration (GUIDE), Department of Pathology & Laboratory Medicine, Section Medical Biology, Laboratory for Tumor Immunology

[2]Division of Molecular Internal Medicine, Department of Internal Medicine II, University Hospital Wuerzburg, Roentgenring 11, 97070 Wuerzburg, Germany

[3]Department of Surgery, Surgical Research Laboratories, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

[4]BioOptical Imaging Center Groningen (BICG), University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

[5]Department of Pharmacy, Therapeutic Gene Modulation, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

*, ± both authors contributed equally

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Abstract

Previously, we demonstrated potent tumor cell-selective pro-apoptotic activity of scFv425:sTRAIL, a recombinant fusion protein comprised of EGFR-directed antibody fragment (scFv425) genetically fused to human soluble TRAIL (sTRAIL). Here we report on the promising therapeutic systemic tumoricidal activity of scFv425:sTRAIL when produced by the replication-deficient adenovirus Ad-scFv425:sTRAIL. *In vitro* treatment of EGFR-positive tumor cells with Ad-scFv425:sTRAIL resulted in the potent induction of apoptosis of not only infected tumor cells, but importantly also of up to 60% of non-infected EGFR-positive tumor cells. A single intraocular injection of Ad-scFv425:sTRAIL in tumor-free nu/nu mice resulted in predominant liver infection and concomitant high blood plasma levels of scFv425:sTRAIL. These mice showed no sign of Ad-scFv425:sTRAIL-related liver toxicity. Identical treatment of mice with established intraperitoneal renal cell carcinoma xenografts resulted in rapid and massive tumor load reduction and subsequent long-term survival. Taken together, adenoviral-mediated *in vivo* production of scFv425:sTRAIL may be exploitable for systemic treatment of EGFR-positive cancer.

Introduction

Adenovirus vectors are attractive gene delivery vehicles for cancer therapy due to their ease of production, high stability *in vivo*, high transgene expression levels, and low pathogenicity in humans. Adenovirus infection is initiated by interaction between carboxy-terminal knob domains of the viral fibers and the extracellular region of the coxsackie/adenovirus receptor (CAR) expressed on the surface of target cells. Unfortunately, CAR is highly expressed on hepatocytes, whereas most cancerous cells only express low levels of CAR. Consequently, intravenously administrated therapeutic adenoviruses predominantly target, infect, and express the transgene payload in the liver [1, 2]. This lack of tumor-selective tropism and strong preference for liver infection has largely excluded the use of therapeutic adenoviruses for systemic treatment of metastatic disease.

To date, strategies to enhance the therapeutic potential of adenovirus have been mainly dedicated to enhancing specificity and efficiency of gene transfer to target cells and the simultaneous untargeting of liver cells [3, 4]. An alternative or complementary approach to enhance the applicability of adenoviruses is the rational design of the payload to be delivered. The aim of this approach is to turn the above-described drawbacks of adenoviruses into an advantage for systemic therapy of metastatic disease. Of potential interest in this respect is the TNF-related Apoptosis Inducing Ligand (TRAIL). The native form of TRAIL is a type II transmembrane protein, but it can also be proteolytically cleaved into soluble homotrimeric TRAIL (sTRAIL) [5, 6]. Recombinant forms of sTRAIL have shown potent tumoricidal activity towards tumor cells of diverse origin and no or minimal adverse activ-

ity towards normal human cells [7-9]. Currently, the tumoricidal activity of sTRAIL is being evaluated in clinical trials [10].

The efficacy of sTRAIL is hampered by the ubiquitous expression of the agonistic receptors TRAIL-R1 and TRAIL-R2 and the antagonistic receptors TRAIL-R3 and TRAIL-R4 on normal tissues. Upon systemic treatment most of the administered sTRAIL will bind to the relative excess of TRAIL-receptors on normal tissues. Consequently, accretion of sTRAIL to tumor cells will be limited. In addition, the therapeutic efficacy of sTRAIL is potentially hampered by its relative inability to activate the high affinity agonistic receptor TRAIL-R2 [11, 12].

Recently, we and others have reported that genetic fusion of sTRAIL to a tumor-selective single chain Fv (scFv) antibody fragment can optimize the efficacy of TRAIL-based therapy [13-19]. Specifically, binding of an scFv:sTRAIL fusion protein to the pre-defined target antigen leads to selective tumor cell accretion. In addition, the soluble and conditionally inactive scFv:sTRAIL is hereby converted into a membrane-bound and active form of TRAIL that can activate both TRAIL-R1 and TRAIL-R2. Importantly, target antigen-bound scFv:sTRAIL can induce bi-/multicellular activation of TRAIL-receptors resulting in apoptotic activity towards proximal tumor cells.

Particularly promising for cancer treatment is fusion protein scFv425:sTRAIL, which selectively targets sTRAIL to human Epidermal Growth Factor Receptor (EGFR)-positive cancer cells [16]. Previously we have shown that scFv425:sTRAIL selectively binds to EGFR-positive cancer cells, whereupon EGFR-mitogenic signaling is rapidly inactivated and cancer cells are sensitized to apoptosis (16). Simultaneously, the sTRAIL domain of EGFR-bound scFv425:sTRAIL potently activates TRAIL-receptor apoptotic signaling (for schematic see Fig. 1).

Here, we investigated the therapeutic potential of scFv425:sTRAIL as payload for adenoviral gene delivery for systemic therapy of metastatic disease. To this end, we generated replication-deficient adenovirus Ad-scFv425:sTRAIL, encoding the cDNA for secreted scFv425:sTRAIL. Treatment of EGFR-positive tumor cells with Ad-scFv425:sTRAIL *in vitro* resulted in potent induction of apoptosis in not only infected but also in >60% of non-infected EGFR-positive tumor cells. Single intravenous injection of Ad-scFv425:sTRAIL in tumor-free nu/nu mice resulted in liver infection and high blood plasma levels of scFv425:sTRAIL in the absence of Ad-scFv425:sTRAIL-related liver toxicity. Identical treatment of mice with established intraperitoneal renal cell carcinoma xenografts resulted in rapid and massive tumor load reduction and subsequent long-term survival. Taken together, adenoviral-mediated *in vivo* production of scFv425:sTRAIL may be exploitable for improved systemic treatment of EGFR-positive carcinoma.

Material and methods

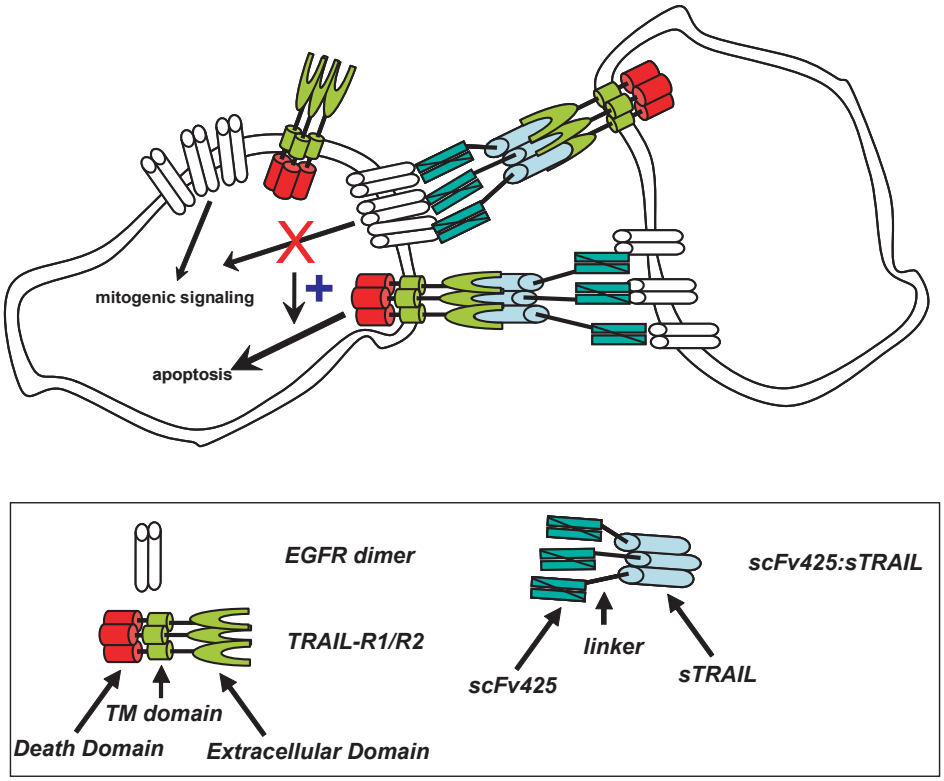

Figure 1. Schematic representation of the mechanism of action of scFv425:sTRAIL. Binding of scFv425:sTRAIL to the Epidermal Growth Factor Receptor (EGFR), via antibody fragment scFv425, inhibits mitogenic signaling of the active EGFR dimer. Consequently, the cell is sensitized to induction of apoptosis by TRAIL. In addition, scFv425-mediated binding immobilizes soluble scFv425:sTRAIL on the cell surface of EGFR-positive tumor cells and converts soluble scFv425:sTRAIL into a membrane bound form. This membrane-bound from of TRAIL can efficiently cross-link and activate the agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2, resulting in the induction of apoptosis. TM; Trans Membrane.

Cell lines

The EGFR-positive renal carcinoma cell line RC21.luc, lentivirally transduced with firefly luciferase, was a generous gift from Prof. Dr. C. Löwik (Leiden University Medical Center, Leiden, The Netherlands). RC21.luc cells were cultured in RPMI 1640 (Cambrex Bio Science, Verviers, France), supplemented with 10% fetal calf serum (FCS). Cells were cultured at 37°C in a humidified 5% CO2 atmosphere.

Primary human hepatocytes (PHH)

Cryopreserved human Hepatocytes (tebu-bio bv, Heerhugowaard, The Netherlands) were isolated using hepatocyte isolation kit (tebu-bio bv, Heerhugowaard, The Netherlands) according to standard protocol. Briefly, vials containing cryopreserved hepatocytes were placed in a shaking water bath (37±1°C) for 1.5 minutes. After thawing, hepatocytes were centrifuged on a Percoll solution (90g, 5 min), the pellet was washed using nutrient rich culture medium and hepatocytes were seeded in a 48 well plate at a density of $0.5x10^6$ cells/ml for experiments.

Mice

Nu/Nu mice were obtained from Harlan (Harlan Netherlands B.V., Horst, The Netherlands). Animals were maintained in a 12h light/dark cycle and fed normal diet and water ad libitum. All animal experiments were approved by the local committee for care and use of laboratory animals and were performed according to governmental and international guidelines on animal experimentation.

Monoclonal antibodies and inhibitors

mAb 425 was kindly provided by Merck (Darmstadt, Germany). mAb425 is a murine IgG2a with a high binding affinity for the extra cellular domain of the EGFR. mAb 425 competes with scFv425 for binding to the EGFR. TRAIL-neutralizing antibody mAb 2E5 was purchased from Alexis (10P, Breda, The Netherlands). Total caspase inhibitor Z-VAD-FMK was purchased from Calbiochem (EMD Biosciences, San Diego, United States).

Construction and production of Ad-scFv425:sTRAIL

Ad-scFv425:sTRAIL was generated by introducing the gene encoding scFv425:sTRAIL [16] into E1- and E3-deleted replication-incompetent recombinant Ad-5 adenovirus using the Ad-Easy system (32). Briefly, cDNA encoding both the κ light chain leader secretion signal and the scFv425:sTRAIL gene were cloned from the previously described vector pEE14-scFv425:sTRAIL [16] into pAdTRACK-CMV using unique HindIII sites. The resulting vector pAdTRACK-CMV-scFv425:sTRAIL was co-transformed with the adenoviral genome vector pAdEasy1 in E. coli BJ5183. After homologous recombination pAd-scFv425:sTRAIL was obtained. Subsequently, pAd-scFv425:sTRAIL was transfected into HEK-293 cells. Adenoviral stock was generated by Cesium Chloride purification, dialyzed in a HEPES-sucrose buffer and kept at -80°C until use. Viral titer was determined by OD260 measurement of viral DNA. Control virus containing luciferase (Ad-Luciferase) was generated essentially as described before (33).

Infection of EGFR-positive tumor cells with Ad-scFv425:sTRAIL

Cells were seeded in 24-well or 96-well plate at 50% confluency and were allowed to ad-

here for 16h. Infection with Ad-scFv425:sTRAIL and the control virus Ad-Luciferase were performed by incubating cells for 24h with the respective adenovirus at the indicated MOI, after which medium was aspirated and fresh medium was added. Analyses were performed 6 days after incubation.

Assessment of tumor cell viability after Ad-scFv425:sTRAIL infection

After infection with Ad-scFv425:sTRAIL, cells were cultured in the presence or absence of mAb 425 (0,5µg/ml), mAb 2E5 (2µg/ml) or total caspase inhibitor Z-VAD-FMK (20µM). Loss of cell viability was assessed by one of the following assays; MTS assay: Loss of viability using the MTS assay was performed according to manufacturer's recommendation (Promega Benelux b.v. Leiden, The Netherlands). Cell viability of medium control was set 100%. Each experimental condition consisted of at least three independent wells. Crystal violet assay: After treatment, crystal violet assay was performed as previously described. This assay gives quantitative information about the relative density of cells adhering to the multi-well dish. The crystal violet stain was solubilised using 1% Sodium Dodecyl Sulfate (SDS) after which staining intensity was quantified by OD550 in a micro-plate reader. Medium control was set at 100% cell survival.

Assessment of apoptosis and loss of cell viability after Ad-scFv425:sTRAIL infection

Apoptosis was assessed by one of the following assays; Loss of mitochondrial membrane potential (Δψ): Δψ was measured by DiOC6-staining (Eugene, The Netherlands) as previously described (19). Briefly, after treatment cells were collected and incubated for 20 min with DiOC6 (0,1 µM) at 37°C, harvested (1000g for 5 min), re-suspended in PBS, and assessed for DiOC6 staining using flow cytometry using an EPICS ELITE flow cytometer (Beckman Coulter, Mijdrecht, The Netherlands). Analysis of apoptotic DNA fragmentation: Apoptotic cleavage of DNA was shown by harvesting total DNA out of cells (2×10^6 cells/well in 6-well plate) 6 days after infection with 100 i.u. AdscFv425:sTRAIL, AdTL or medium control. Subsequent isolation of total DNA was performed by use of the DNA isolation kit DNeasy Tissue Kit (QIA-GEN, the Netherlands). Equal amounts of DNA were separated by electrophoresis on a 1,6% agarose gel and visualized under UV-light after ethidium bromide staining.

Induction of apoptosis by secreted scFv425:sTRAIL in non-infected EGFR-positive tumor cells

To accurately assess the anti-tumor effect of secreted scFv425:sTRAIL, a trans-well system was used in which RC21.luc cells were cultured in the lower well and infected with Ad-scFv425:sTRAIL or Ad-TL. Infected cells were separated from cell culture inserts, containing non-infected RC21.luc cells, by a polycarbonate membrane with pore size of 0,4

µm (NUNC, Roskilde, Denmark), which allows free diffusion of the scFv425:sTRAIL fusion protein, but not of Ad-scFv425:sTRAIL virus particles.

Production and secretion of scFv425:sTRAIL in nu/nu mice

Nude mice were intra-occularly injected with saline control, Ad-luciferase (10^{10}), Ad-scFv425:sTRAIL (10^{9}), Ad-scFv425:sTRAIL (10^{10}). After 3 days, mice were sacrificed, plasma was collected and liver samples were analyzed for presence of activated caspase-3/-7. Briefly, equal mass of liver samples were analyzed for caspase-3/-7 activity using the Caspase-Glo 3/7 Assay according to manufacturer's recommendations (Promega). As positive control for liver toxicity mice were treated with Flag-FasL + anti-Flag mAb.

Isolated plasma was analyzed for scFv425:sTRAIL presence using TRAIL-ELISA according to manufacturer's instructions (Diaclone SAS, Besançon, France). Plasma samples were also tested for tumoricidal activity in vitro by treatment of RC21.luc cells for 16 h *in vitro*, after which apoptosis was assessed by Δψ.

Tissue preparation and histology

Livers were explanted from sacrificed mice at day 35 (saline and Ad-scFv425:sTRAIL 10^{9} vp treated mice) and at day 64 (Ad-scFv425:sTRAIL 10^{10} vp treated mice) and immediately snap-frozen in liquid nitrogen. Five µm sections were cut from cryofixed tissues, thawed and fixed in acetone. H&E staining was performed to assess the histology of livers according to standard protocol.

RC21.luc i.p. xenograft model in nu/nu mice

RC21.luc cells ectopically expressing luciferase were intraperitoneally injected ($2x10^{6}$/mice) in nude/nude mice. Tumor growth was monitored using bioluminescent imaging as described in more detail below. After hatching of the xenograft, mice were treated with 1. saline, 2. Ad-scFv425:sTRAIL (10^{9}), 3. Ad-scFv425:sTRAIL (10^{10}), respectively. The group size of saline and Ad-scFv425:sTRAIL (10^{9}) was 3. The group size of Ad-scFv425:sTRAIL (10^{10}) was 4.

Response to treatment was measured using bioluminescent imaging. At various time-points, blood samples were taken from the mice to allow for detection of scFv425:sTRAIL production. Control treated mice and Ad-scFv425:sTRAIL (10^{9}) were sacrificed at day 32 after start of treatment. Mice treated with Ad-scFv425:sTRAIL (10^{10}) remained healthy up to day 64, after which mice were terminated. At termination, blood was collected for endpoint reading of scFv425:sTRAIL concentration.

In vivo bioluminescence imaging of luciferase expression of RC21.luc xenografts

Bioluminescence imaging was performed using an ultra-sensitive charge-coupled device

(CCD) camera cooled to -105°C, within the In Vivo Imaging System IVIS®100 (Xenogen, Alameda, CA, USA). An aqueous solution of beetle D-luciferin (150 mg/kg; Xenogen) was injected intraperitoneally 10 minutes before imaging. Mice were placed into the light-tight chamber of the CCD camera imaging system in anesthetized condition (2.5% isoflurane in oxygen 1.5 L/min). The mice were imaged from the ventral abdominal side and a digital grayscale reference image was taken. Subsequently, the presence and amount of RC21.luc cells was assessed by detecting photons emitted by RC21.luc cells that were transmitted through the body tissue. The Living Image®2.50 software program (Xenogen, Alameda, CA) was used for data acquisition and analysis. This program was used to create a pseudocolor image. The luminescence image was merged with the reference image to create an overlay image that enables anatomical localization. Images were displayed and quantified in log radiance (photons/sec/cm2/sr) enabling absolute comparison between bioluminescent images between different treatment groups. Region-of-interest (ROI) measurements, used to quantify the amount of light emission in specific areas of the image, were defined to determine the maximum emission from the transfected cells. ROI size and shape was kept identical along longitudinal measurements for each subject.

Results

Ad-scFv425:sTRAIL-infection leads to EGFR-restricted induction of apoptosis in infected and non-infected EGFR-positive tumor cells

Previously we demonstrated EGFR-restricted and TRAIL-mediated apoptotic activity of scFv425:sTRAIL [16]. To assess whether virally produced scFv425:sTRAIL similarly induced apoptosis in EGFR-positive tumor cells, RC21.luc cells were infected with Ad-scFv425:sTRAIL. The viability of RC21.luc cells was strongly reduced with increasing Multiplicity of Infection (MOI) (Fig. 2a). This loss of viability increased in time after infection, from 45% at day 1 to 60% at day 6 post-infection (Fig. 2b). Loss of viability was accompanied by typical apoptotic DNA-fragmentation (Fig. 2c). In contrast, infection with control adenovirus encoding for luciferase (Ad-Luciferase) did not reduce viability nor induce DNA-fragmentation (Fig. 2a-c). Importantly, induction of apoptosis by scFv425:sTRAIL was abrogated by co-incubation with molar excess of EGFR-blocking mAb 425, as well as by a TRAIL-neutralizing antibody or by total caspase inhibitor zVAD-FMK (Fig. 2d). Together, this indicates that virally produced scFv425:sTRAIL potently activates TRAIL-mediated apoptosis only upon specific binding to cell surface expressed EGFR.

EGFR-specific binding of secreted scFv425:sTRAIL and subsequent bi-/multicellular activation of TRAIL-receptors provides scFv425:sTRAIL with apoptotic activity towards non-infected EGFR-positive tumor cells. To evaluate the effect towards non-infected cells, Ad-scFv425:sTRAIL infected cells and non-infected RC21.luc cells were co-cultured, while being separated by a membrane impermeable to adenovirus but permeable to soluble

scFv425:sTRAIL. In these experiments, >60% of non-infected RC21.luc cells were eliminated by apoptosis (Fig. 2e). In similar experiments with Ad-luciferase (MOI 100), no apoptosis was induced (Fig. 2e). Taken together, these data demonstrate that virally produced and secreted scFv425:sTRAIL efficiently activates apoptosis in not only infected but also in

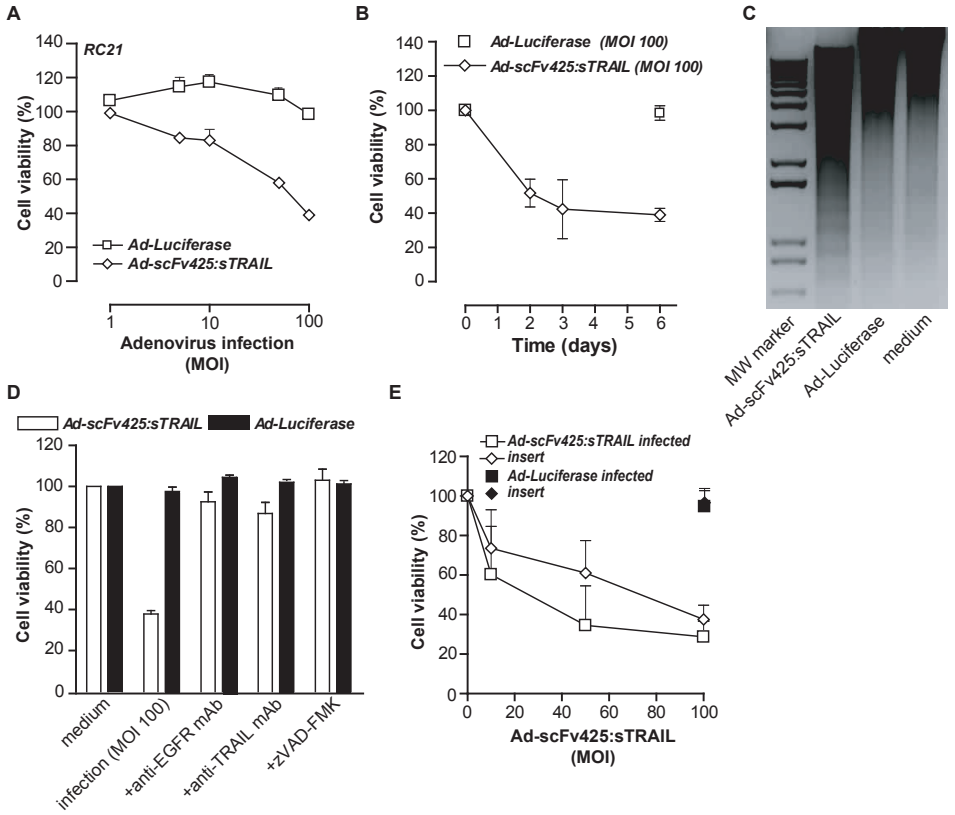

Fig. 2. EGFR-restricted induction of apoptosis by adenovirally produced scFv425:sTRAIL. **A.** RC21.luc cells were infected Ad-scFv425:sTRAIL or Ad-Luciferase at varying MOIs. At 6 days post-infection, loss of viability was quantified by MTS assay. **B.** RC21 cells were infected with Ad-scFv425:sTRAIL or Ad-Luciferase (MOI 100), after which cell viability was determined at various time-points. **C.** RC21.luc cells were infected with Ad-scFv425:sTRAIL or Ad-Luciferase (MOI 100), after which DNA was isolated at day 6 of infection and evaluated for apoptotic DNA-fragmentation. **D.** Supernatant produced by Ad-scFv425:sTRAIL infected RC21.luc cells was used to treat non-infected RC21.luc cells in the presence or absence of TRAIL-neutralizing mAb 2E5 or EGFR-blocking mAb 425. **E.** RC21.luc cells were seeded in a trans-well system, after which cells in the lower compartment were treated with Ad-scFv425:sTRAIL at varying MOI or Ad-Luciferase at MOI 100. After 6 days, induction of apoptosis in both infected and non-infected RC21.luc target cells was analyzed by MTS assay. Values are representatives of 3 independent experiments.

non-infected EGFR-positive tumor cells.

Infection of nude/nude mice with Ad-scFv425:sTRAIL yields high plasma levels of scFv425:sTRAIL in the absence of liver toxicity

To determine whether infection with Ad-scFv425:sTRAIL resulted in production and secretion of scFv425:sTRAIL *in vivo* by hepatocytes, nude mice were infected by intra-ocular injection with Ad-scFv425:sTRAIL (10^9 or 10^{10} viral particles). Infection with a control adenovirus encoding for luciferase (Ad-Luciferase) confirmed that predominantly liver cells were infected, as evidenced by the strong exclusive luminescent signal in the liver (Fig. 3a). At 3 days post-infection, infection with 10^{10} vp Ad-scFv425:sTRAIL resulted in high plasma levels of >10 µg/ml scFv425:sTRAIL (Fig. 3b; 11.2+/-8 µg/ml). In contrast, infection with 10^9 viral particles (vp) Ad-scFv425:sTRAIL yielded only minimal plasma concentrations of scFv425:sTRAIL (Fig. 3b; 60+/-20 ng/ml).

Subsequent *in vitro* treatment of RC21.luc cells with plasma isolated from the various treatment groups revealed that only plasma obtained from mice infected with 10^{10} vp of Ad-scFv425:sTRAIL potently induced apoptosis (Fig. 3c). Induction of apoptosis by scFv425:sTRAIL was still dependent on scFv425-mediated binding to EGFR, since co-incubation with parental mAb 425 (20µg/ml) strongly inhibited the induction of apoptosis. Moreover, apoptosis was fully TRAIL-mediated, since co-incubation with TRAIL-neutralizing antibody abrogated apoptosis (Fig. 3d).

No toxicity was detected upon visual inspection of the isolated livers (illustrated in Fig. 4a) and no effector caspase activity (caspase-3/-7) was detected in livers of mice infected with 10^{10} vp of Ad-scFv425:sTRAIL (Fig. 4b). In contrast, treatment with cross-linked sFasL induced strong toxicity with high levels of active caspase-3/-7 (Fig. 4b). Importantly, histological analysis confirmed the absence of toxicity after adenoviral infection (no centrilobular necrosis, cholestasis, hepatocyte injury, fatty changes, hepatitis), with liver morphology identical to saline treated mice (Fig. 5a-c). Together, these data indicate that infection with 10^{10} vp Ad-scFv425:sTRAIL yields high plasma levels of active scFv425:sTRAIL in the absence of liver toxicity.

To further determine the effect of Ad-scFv425:sTRAIL infection on human hepatocytes, we performed an initial characterization by infecting primary human hepatocytes (PHH). Importantly, infection with Ad-scFv425:sTRAIL only minimally reduced cell viability 3 days post-infection (Fig. 5d). Since PHH in culture are known to dedifferentiate, we additionally assessed the immediate effect of fusion protein scFv425:sTRAIL, by treating PHH with plasma of mice infected with 10^{10} vp Ad-scFv425:sTRAIL for 16h. Importantly, no significant induction of apoptosis was detected (Fig. 5e). In contrast, treatment with killer-FasL, a known inducer of apoptosis in hepatocytes, strongly reduced viability after 3 days and induced apoptosis after 24h (Fig. 5d and e). Together, these preliminary results indicate

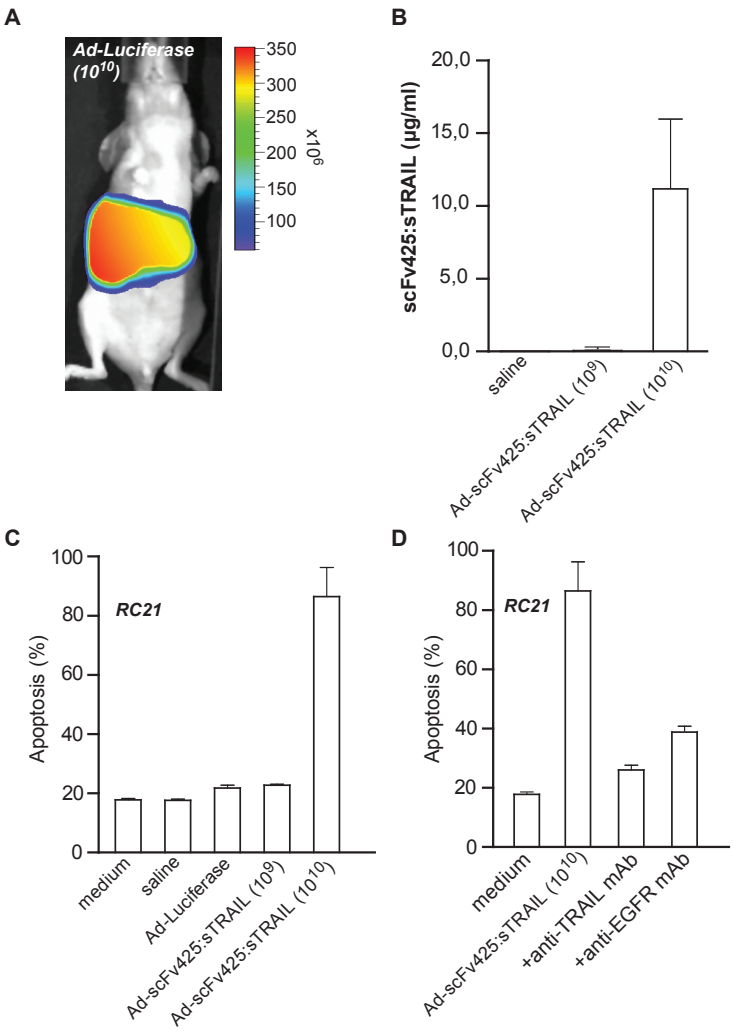

Figure 3. Efficient production and secretion of scFv425:sTRAIL upon Ad-scFv425:sTRAIL infection of nude mice. A. Mice (n=3) were infected with control adenovirus Ad-Luciferase and were imaged for bioluminescence 3 days post-infection. A representative picture of bioluminescence (ph/sec/cm2/sr) after Ad-Luciferase infection is given. **B.** Mice were infected with saline or low and high dose of Ad-scFv425:sTRAIL (10^9 and 10^{10}vp, respectively). After 3 days, concentration of scFv425:sTRAIL in plasma was determined by TRAIL ELISA. **C.** RC21.luc cells were treated with plasma obtained from mice treated with saline, Ad-Luciferase, Ad-scFv425:sTRAIL (10^9vp) or Ad-scFv425:sTRAIL (10^{10}vp). After 16h, induction of apoptosis was assessed by Δψ. **D.** RC21.luc cell were treated with plasma obtained from mice infected with high dose Ad-scFv425:sTRAIL in the presence or absence of TRAIL-neutralizing mAb 2E5 or EGFR-blocking mAb 425. Apoptosis was assessed by Δψ.

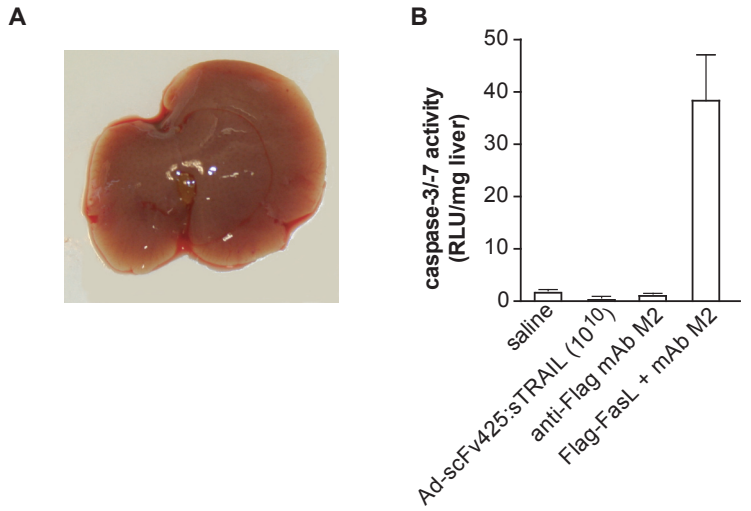

Figure 4. No detectable liver damage after infection with Ad-scFv425:sTRAIL. A. A representative picture of an isolated liver after Ad-scFv425:sTRAIL (10^{10} vp) infection. **B.** Isolated livers of saline, low and high dose Ad-scFv425:sTRAIL treated mice were analyzed for caspase-3/-7 activity. As positive control for liver caspase-3-7 activation, mice were treated with Flag-FasL secondarily cross-linked with anti-Flag mAb M2. Values are represented as relative light units per mg of liver.

that Ad-scFv425:sTRAIL infection is not associated with human hepatocyte apoptosis.

scFv425:sTRAIL eradicates established RC21.luc xenografts in nude/nude mice

To asses the tumoricidal activity of Ad-scFv425:sTRAIL, nu/nu mice with established intra-peritoneal RC21.luc xenografts were infected with Ad-scFv425:sTRAIL (10^9 vp) or Ad-scFv425:sTRAIL (10^{10} vp) by intraocular injection. The xenografted RC21.luc cells express firefly luciferase, allowing for real-time *in vivo* imaging of tumor size and response to therapy. In saline treated mice, RC21.luc xenografts continued to grow rapidly until mice had to be sacrificed at day 32 (Fig. 6a). Compared to saline-treated mice, already a significant inhibition in tumor growth was detected in mice treated with 10^9 vp of Ad-scFv425:sTRAIL (Fig. 6b). However, injection with 10^{10} vp of Ad-scFv425:sTRAIL resulted in a rapid and dramatic reduction in tumor size (Fig. 6c). All 10^{10} vp Ad-scFv425:sTRAIL-treated mice remained healthy until the end of the experiment.

Conversion of the quantitative bioluminescent imaging data into a relative percentage of tumor size compared to start of treatment revealed that in saline treated mice tumors grew exponentially to ~3500% of initial size (Fig. 7a). Upon treatment with low dose of Ad-scFv425:sTRAIL (10^9 vp), mice showed retarded tumor growth. In these mice, tumor growth was markedly slower than in saline-treated mice (Fig. 6a, day 32: Ad-scFv425:sTRAIL (10^9

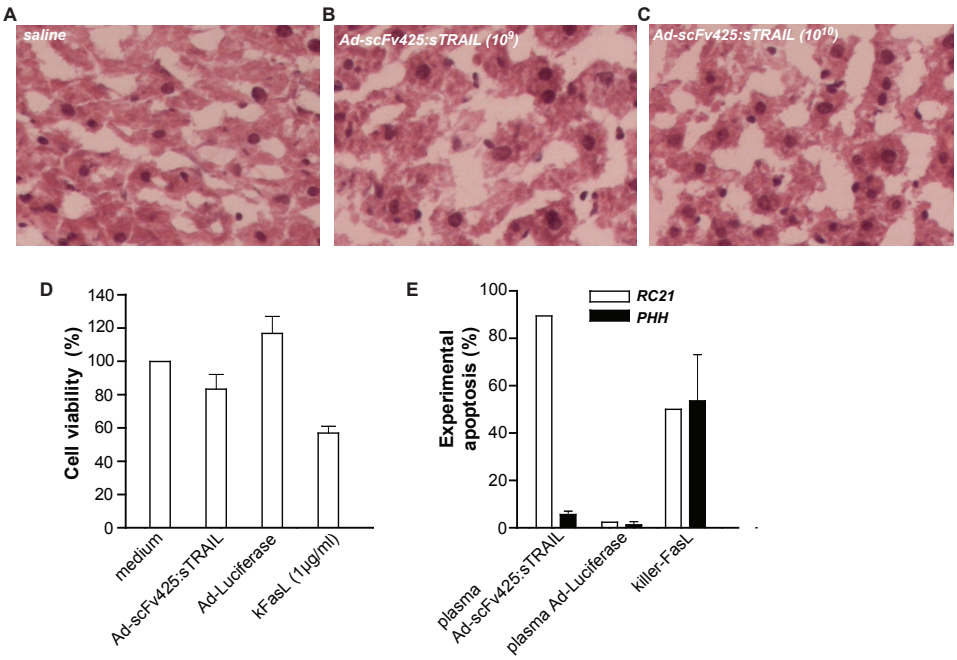

Figure 5. No detectable liver damage after infection with Ad-scFv425:sTRAIL. Normal liver histology, as determined by H&E staining upon saline treatment (**A**), Ad-scFv425:sTRAIL (10^9 vp) treated (**B**) and in Ad-scFv425:sTRAIL (10^{10} vp) treated mice (**C**). Original magnification 200x. **D**. PHH were infected with Ad-scFv425:sTRAIL (MOI 100), Ad-Luciferase (MOI 100) or treated with killer-FasL (5µg/ml). Viability 3 days after infection was measured using MTS assay. **E**. PHH were treated for 16h with plasma obtained from mice infected with Ad-scFv425:sTRAIL (10^{10} vp), yielding a concentration of 5µg/ml, saline control or killer-FasL (5µg/ml). Apoptosis was assessed by Δψ.

vp); 900% of initial size, saline; 3500% of initial size). Importantly, in mice treated with 10^{10} vp of Ad-scFv425:sTRAIL a dramatic reduction of 97% of initial tumor size occurred within days of treatment (Fig. 7a). Two of the four mice retained a minimal residual tumor that corresponded to ~2% of initial tumor size until the end of the experiment at day 64.

Of note, the conversion of the raw bioluminescent data of Fig 6 to a relative percentage of tumor size in Fig 7a reveals a very important aspect of the data, namely, that the increase or decrease in tumor size after treatment is remarkably constant for each treatment group. Therefore, the effect of the respective treatments appears robust and reproducible along the range of variable tumor sizes detected in the mice.

In close agreement with the above experiment in tumor free mice, plasma scFv425:sTRAIL levels in Ad-scFv425:sTRAIL (10^9 vp) treated mice reach a peak plasma level of only 12 ng/ml scFv425:sTRAIL (Fig. 7b). Thus, this very low concentration of scFv425:sTRAIL already

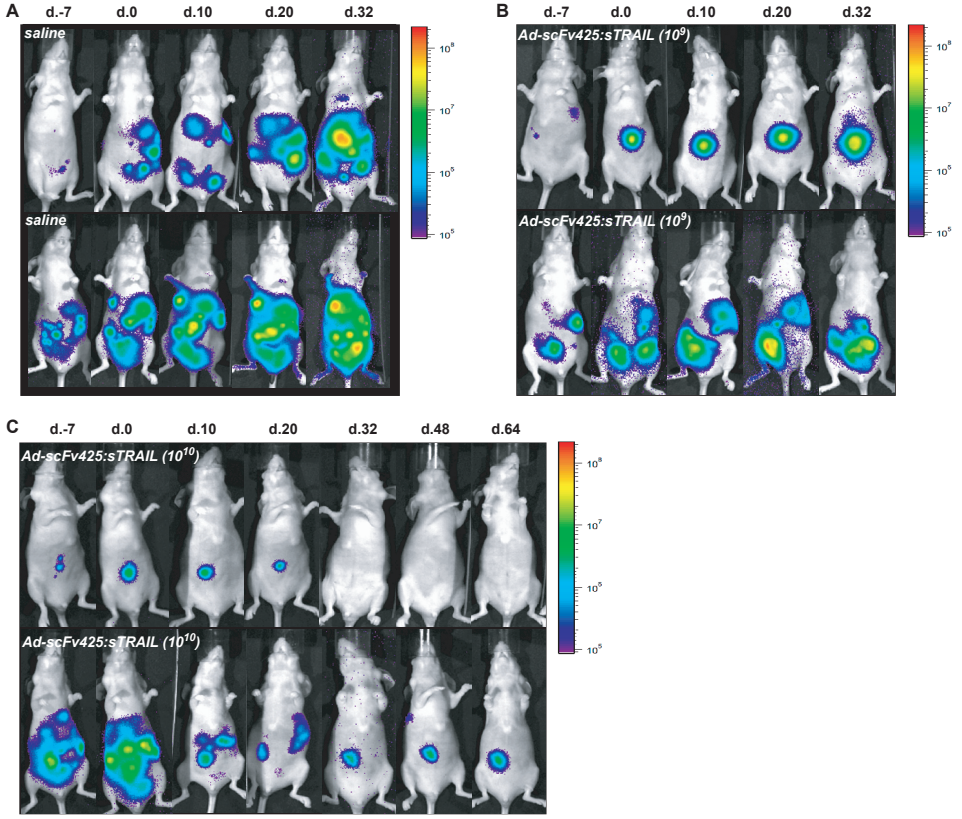

Figure 6. Ad-scFv425:sTRAIL infection eradicates established RC21.luc xenografts in nude mice. Nude mice with established i.p. RC21.luc xenografts were treated with (**A**) saline, (**B**) Ad-scFv425:sTRAIL (10^9vp), (**C**) Ad-scFv425:sTRAIL (10^{10}vp). Tumor size was monitored real-time using BLI. The luminescence image was merged with the reference image to create an overlay image that enables anatomical localization. Images were displayed and quantified in log radiance (photons/sec/cm2/sr), enabling absolute comparison between bioluminescent images between different treatment groups. Mice treated with saline and low dose Ad-scFv425:sTRAIL were sacrificed after 32 days. Mice treated with high dose of Ad-scFv425:sTRAIL were sacrificed at day 64. The two panels in a, b and c show the variability in tumor size within each treatment group at the start of treatment.

significantly delayed the outgrowth of RC21.luc xenografts from 3500% to 900% at the time of sacrifice. In mice infected with 10^{10} vp Ad-scFv425:sTRAIL, plasma scFv425:sTRAIL concentrations proved to be very high, with a peak level of ~380 µg/ml 7 days post-infection (Fig. 7c).

In the minimal residual tumors of mice treated with 10^{10} vp Ad-scFv425:sTRAIL, there was an apparent equilibrium between tumor cell proliferation and induction of apoptosis

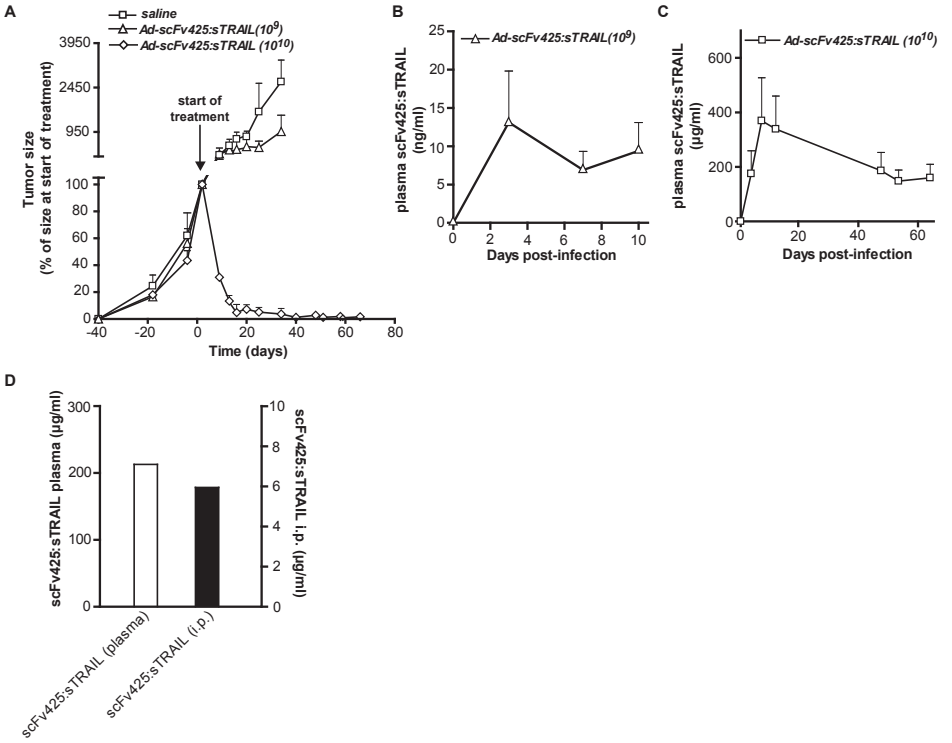

Figure 7. Ad-scFv425:sTRAIL infection eradicates established RC21.luc xenografts in nude mice. A. Graph of tumor size in mice treated with saline, Ad-scFv425:sTRAIL (10^9vp) and Ad-scFv425:sTRAIL (10^{10}vp). Tumor size was monitored using BLI and is represented as relative size compared to tumor size at start of treatment. **B**. Concentration of plasma scFv425:sTRAIL in mice treated with Ad-scFv425:sTRAIL (10^9vp). **C**. Concentration of plasma scFv425:sTRAIL in mice treated with Ad-scFv425:sTRAIL.(10^{10}vp) **D**. Concentration of scFv425:sTRAIL in plasma and i.p. fluid in mice treated with Ad-scFv425:sTRAIL (10^9vp) at day 64 post-infection. In B-D, TRAIL concentrations were determined using TRAIL ELISA.

by scFv425:sTRAIL, since the luminescent signal remained constant until the end of the experiment. Based on the small size of the tumors, we wondered whether the activity of scFv425:sTRAIL might be hampered by poor tumor vascularization. In the absence of vasculature, scFv425:sTRAIL would first need to diffuse over the peritoneal membrane and then into the tumor. Although suggestive, in one of the mice that retained an RC21.luc xenograft the diffusion of scFv425:sTRAIL into the intra-peritoneal fluid proved to yield an >30 fold lower concentration scFv425:sTRAIL compared to plasma concentrations (Fig. 7d), indicating that intra-peritoneal and subsequent intra-tumoral diffusion might be a rate-limiting step for scFv425:sTRAIL activity.

Discussion

Previously we reported on the pro-apoptotic activity of the EGFR-targeted fusion protein scFv425:sTRAIL. Here, we show that combining this therapeutic approach with the typical characteristics of adenoviral gene therapy yields promising pro-apoptotic activity in vitro and exerts potent tumoricidal activity *in vivo*. Our *in vitro* experiments demonstrate that virally induced production of scFv425:sTRAIL leads to apoptotic activity according to the intended mechanism of action. Specifically, antibody-fragment mediated binding to EGFR results in cell surface accretion of scFv425:sTRAIL and concomitant potent induction of apoptosis by the now fully active sTRAIL domain. Importantly, scFv425:sTRAIL exerts its pro-apoptotic activity by binding to cell surface expressed EGFR and TRAIL-receptors (for schematic see Fig. 1). As a result, scFv425:sTRAIL induces apoptosis in infected cells and in non-infected cells with equal efficiency.

The mechanism of action of scFv425:sTRAIL endows this fusion protein with superior qualities for systemic gene therapeutic approaches. In particular, virally expressed scFv425:sTRAIL is efficiently secreted and inactive towards normal human cells. Moreover, scFv425:sTRAIL induces apoptosis upon selective binding to EGFR on the cell surface of targeted tumor cells only. Therefore, there is no need for a direct infection of tumor cells or, for that matter, for a complete infection of all tumor cells. This is in contrast to most gene therapeutic strategies aimed at the delivery of toxic moieties. The safety and efficacy of these approaches critically depends on the restricted infection of tumor cells only, in order to prevent toxicity towards normal human cells [2, 20]. Moreover, gene therapy with e.g. pro-drug converting enzymes (GDEPT) relies on the intracellular production of toxic metabolites in the cytoplasm of the infected cell that then need to diffuse to non-infected bystander cells [21]. Usually, these toxic metabolites cannot freely transit the cell membrane but require e.g. gap-junctional inter-cellular communication (GJIC) [22]. Unfortunately, most cancer cells lack functional GJIC, thereby largely restricting the tumoricidal activity to infected cells.

For systemic therapy, Ad-scFv425:sTRAIL infection should be followed by the correct and high level production, subsequently yielding high blood plasma levels of scFv425:sTRAIL. An initial *in vivo* assessment of scFv425:sTRAIL production after intra-ocular injection with 10^{10} viral particles Ad-scFv425:sTRAIL revealed high plasma concentrations of up to 10 µg/ml. Interestingly, infection with only 10-fold lower concentration of Ad-scFv425:sTRAIL yielded only plasma levels of ~60 ng/ml fusion protein. Such marked differences in plasma concentrations relative to viral titer have been described previously and were attributed mainly to adenoviral particle clearance by liver Kupffer cells [23], most likely by scavenger receptors [24]. Our control *in vivo* bioluminescent imaging experiments with Ad-luciferase confirmed that predominantly liver cells were infected upon intra-ocular injection. Together, this indicates that at lower MOI all viral particles are taken up by liver Kupffer cells and

eliminated, whereas at higher MOI the Kupffer cells are saturated and hepatocytes are infected.

Importantly, the µg/ml range of scFv425:sTRAIL produced in the liver after infection with 10^{10} viral particles Ad-scFv425:sTRAIL is not associated with obvious liver damage, as evidenced by a normal healthy appearance of isolated livers, absence of signs of hepatotoxicity, the absence of liver caspase-3/-7 activity, as well as the minimal activity on primary cultured human hepatocytes. Taken together, the efficient production and lack of toxicity confirm the potential of Ad-scFv425:sTRAIL for systemic adenoviral therapy.

Subsequent xenograft studies with Ad-scFv425:sTRAIL revealed promising tumoricidal activity of systemically produced fusion protein. Infection with 10^{9} viral particles of Ad-scFv425:sTRAIL, yielding only low ng/ml scFv425:sTRAIL blood plasma levels, retarded tumor outgrowth from 3500% to 900% at 32 days after infection. Apparently, this low systemic concentration is already sufficient to significantly inhibit tumor growth. Intriguingly, tumor growth started to diverge only at later time-points, from approximately 25 days after start of treatment. Upon increasing tumor size, the rate of growth of tumor will increasingly rely on neovascularization. At the same time, increased vascularization will optimize infusion of scFv425:sTRAIL to the tumor cells. Together, this suggests that the divergence in tumor growth at later time-points may be due to an increase in neovascularization and consequently increased access of scFv425:sTRAIL to tumor cells.

After infection with 10^{10} viral particles two of the four mice achieved a complete remission. In the two other mice, there was a rapid and dramatic reduction in tumor size, which in time was followed by the continued presence of a minimal residual tumor. Importantly, these minimal residual tumors retained an identical bioluminescent signal from day 20 up to day 64 post-infection, indicating that tumor growth was completely halted. Apparently, there was a balance between proliferation of tumor cells and the induction of apoptosis by scFv425:sTRAIL.

Small tumor nodules of 1-2 mm in diameter are in the so-called avascular phase and do not require neovascularization for survival [25]. Based on the apparent equilibrium between proliferation and apoptosis in the minimal residual tumors, we wondered whether the tumoricidal activity of scFv425:sTRAIL might be hampered by a lack of vascularization or poor vascularization. In the absence of vasculature, scFv425:sTRAIL can only diffuse into the tumor from the intraperitoneal fluid. Circumstantial evidence for this hypothesis can be found from the fact that the intra-peritoneal concentration of scFv425:sTRAIL proved to be 30-fold lower than the plasma concentration of scFv425:sTRAIL. This finding suggests that diffusion of scFv425:sTRAIL from plasma to the intraperitoneal fluid and from the intraperitoneal fluid into the tumor might indeed be a limiting step for the tumoricidal activity.

From our data it is clear that without direct infection of tumor cells, Ad-scFv425:sTRAIL yields potent tumoricidal activity towards established renal cell carcinoma xenografts, this

in contrast to e.g. immunotoxin-based gene therapeutic strategies, where it is imperative to solely infect targeted tumor cells. Obviously, systemic infection with Ad-scFv425:sTRAIL can be further combined with tumor localized production of scFv425:sTRAIL by intra-tumoral injection of Ad-scFv425:sTRAIL or by incorporating the adenovirus retargeting strategies that are being developed by other researchers. For instance, Ad-scFv425:sTRAIL can be retargeted to specifically infect EGFR-positive tumor cells using bispecific antibody 425:S11 [24] or alternatively to any other carcinoma-associated antigen [26].

An advantageous feature of using TRAIL as payload is the fact that the pro-apoptotic activity of TRAIL is synergized by a wide variety of other therapeutic approaches, including novel drugs such as EGFR-tyrosine kinase inhibitor Iressa and proteasome inhibitor velcade [16, 27]. Of particular interest in the context of adenoviral delivery of TRAIL-based therapeutics is the histone deacetylase inhibitor (HDACi) Valproic Acid (VPA). Recently, VPA was shown to enhance the efficacy of adenoviral infection due to upregulation of the CAR receptor [28], which may help to increase infection of hepatocytes at lower doses of adenovirus. In this respect, further combination with inhibition of uptake and concomitant degradation of adenovirus by Kupffer cells using polyinosinic acid, as recently described by us [24], may be a strategy worthwhile pursuing. Taken together with the established synergistic activity of combined treatment of tumor cells with HDACi and TRAIL [19, 29], VPA may be an ideal candidate to both optimize the efficiency of Ad-scFv425:sTRAIL infection as well as the tumoricidal activity of scFv425:sTRAIL.

In conclusion, our data clearly show that systemic infection with Ad-scFv425:sTRAIL results in potent tumoricidal activity towards EGFR-positive human carcinoma xenografts. Adenoviral-mediated *in vivo* production of scFv425:sTRAIL and possibly other scFv:sTRAIL fusion proteins may be exploitable for improved systemic treatment of cancer.

Acknowledgements

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Targeted delivery of a designed sTRAIL mutant results in superior apoptotic activity towards EGFR-positive tumor cells.

Edwin Bremer, Marco de Bruyn, Douwe Samplonius, Theo Bijma, Bram ten Cate, Lou de Leij and Wijnand Helfrich

Groningen University Institute for Drug Exploration (GUIDE), Department of Pathology & Laboratory Medicine, Section Medical Biology, Laboratory for Tumor Immunology, University Medical Center Groningen, University of Groningen, The Netherlands.

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Abstract

Previously, we have shown that EGFR-selective delivery of sTRAIL, by genetic fusion to antibody fragment scFv425, enhances the tumor-selective pro-apoptotic activity of sTRAIL. Insight into the respective contribution of the agonistic receptors TRAIL-R1 and TRAIL-R2 to TRAIL-induced apoptosis may provide a rational approach to further optimize TRAIL-based therapy. Recently, this issue has been investigated using sTRAIL mutants designed to selectively bind to either receptor. However, the relative contribution of the respective TRAIL receptors, in particular TRAIL-R1, in TRAIL-signaling is still unresolved. Here, we fused scFv425 to designed sTRAIL mutant sTRAILmR1-5, reported to selectively activate TRAIL-R1, and investigated the therapeutic apoptotic activity of this novel fusion protein. EGFR-specific binding of scFv425:sTRAILmR1-5 potently induced apoptosis, which was superior to the apoptotic activity of scFv425:sTRAIL-wt and a non-targeted MOCK-scFv:sTRAILmR1-5. During co-treatment with cisplatin or the histone deacetylase inhibitor VPA, scFv425:sTRAILmR1-5 retained its superior pro-apoptotic activity compared to scFv425:sTRAIL-wt. However, in catching-type ELISAs with TRAIL-R1:Fc and TRAIL-R2:Fc, scFv425:sTRAILmR1-5 was found to not only bind to TRAIL-R1 but also to TRAIL-R2. Binding to TRAIL-R2 also had functional consequences, since the apoptotic activity of scFv425:sTRAILmR1-5 was strongly inhibited by a TRAIL-R2 blocking monoclonal antibody. Moreover, scFv425:sTRAILmR1-5 retained apoptotic activity upon selective knockdown of TRAIL-R1 using small inhibitory RNA. Collectively, these data indicate that both agonistic TRAIL-receptors are functionally involved in TRAIL-signaling by scFv425:sTRAILmR1-5 in solid tumor cells. Moreover, the superior target cell-restricted apoptotic activity of scFv425:sTRAILmR1-5 indicates its therapeutic potential for EGFR-positive solid tumors.

Introduction

The Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) is normally present as a type II transmembrane protein on immune effector cells, such as NK-cells. As such, TRAIL is involved in the elimination of e.g. metastasizing cancer cells (1,2). TRAIL can also be proteolytically cleaved into a homotrimeric soluble form (sTRAIL) that partly retains tumoricidal activity (3,4). Several recombinant derivatives of sTRAIL have been generated that all display promising anti-tumor activity *in vitro* and in human xenografted tumor mouse models (5,6,7).

Recently, we and others have demonstrated that the tumor-cell specific activity of sTRAIL can be augmented by genetic fusion to a tumor-selective antibody fragment (8,9,10,11,12). Antibody fragment-mediated binding of such scFv:sTRAIL fusion proteins to a cell surface-expressed target antigen results in tumor cell accretion and converts soluble TRAIL into

membrane-bound TRAIL. Subsequently, agonistic TRAIL receptors are efficiently activated in a monocellular and/or bicellular manner.

TRAIL signals apoptosis by binding to the agonistic receptors TRAIL-R1 and TRAIL–R2 (13,14,15). Concomitantly, an intracellular cascade of caspase activation ensues that ultimately results in the apoptotic demise of the cell. These agonistic receptors are characterized by a cytoplasmic region known as the Death Domain, which is critical for signal transduction upon TRAIL-binding. TRAIL can also interact with three antagonistic receptors, TRAIL-R3, TRAIL-R4 and osteoprotegerin (OPG). TRAIL-R3 is a phospholipid-anchored receptor that lacks a cytoplasmic domain (16,14,17). TRAIL-R4 has a truncated intracellular domain incapable of transmitting the apoptotic signal (18,19,20). Osteoprotegerin is a soluble receptor for TRAIL (21) that is best known for its involvement in bone homeostasis as a soluble receptor for the TNF homolog RANKL.

This intricate receptor system, with five distinct receptors that differentially bind and interact with TRAIL, suggests that the outcome of TRAIL-signaling is subject to a high degree of regulation (22). Therefore, insight into the respective contribution of the agonistic receptors TRAIL-R1 and TRAIL-R2 to apoptotic signaling by TRAIL may provide a rational approach to optimize TRAIL therapy for a specific tumor type.

Several laboratories have studied this issue using sTRAIL mutants designed to selectively bind to one of the agonistic TRAIL-receptors and not to the antagonistic receptors (23,24,25). Using TRAIL-R1 and TRAIL-R2 selective sTRAIL mutants Kelley et al. ascribed a greater contribution of TRAIL-R2 to TRAIL-apoptotic signaling in solid tumor cells (23). Similarly, van der Molen et al. reported that selective TRAIL-R2 activation results in enhanced pro-apoptotic activity (24). On the other hand, MacFarlane et al. concluded that apoptosis signaling was exclusively mediated by TRAIL-R1 in Chronic Lymphocytic Leukemia (25). Importantly, experimental data in the latter paper indicates that the TRAIL-R1 selective sTRAIL mutant used by Kelley et al. is actually largely inactive. Thus, the exact contribution of TRAIL-R1 and TRAIL-R2 to TRAIL-induced apoptosis remains to be elucidated.

Previously, we have demonstrated that EGFR-targeted delivery of wild-type sTRAIL, using scFv425:sTRAIL, enhanced the tumor-selective binding and activity (10). Here, we genetically fused antibody fragment scFv425 to sTRAILmR1-5, reported by MacFarlane et al. to selectively activate TRAIL-R1, and investigated the therapeutic apoptotic activity of this novel fusion protein. Fusion protein scFv425:sTRAILmR1-5 showed superior apoptotic activity compared to the corresponding wild-type sTRAIL fusion protein on half of the EGFR-positive solid tumor cell lines tested and showed a non-significant trend to higher apoptotic activity on the other cell lines. Furthermore, scFv425:sTRAILmR1-5 showed superior apoptotic activity in comparison to a MOCK-scFv:sTRAILmR1-5 fusion protein, with irrelevant target specificity. However, in contrast to the findings of MacFarlane et al. we

found that the sTRAILmR1-5 domain in our scFv425:sTRAILmR1-5 fusion protein also bound to and partly signaled apoptosis via TRAIL-R2. Taken together, EGFR-selective delivery and induction of apoptosis by scFv425:sTRAILmR1-5 is a potentially promising therapeutic approach for EGFR-positive solid tumors.

Materials and methods

Cell lines

The following cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, USA); Jurkat (ALL T-cell line), Ramos (Burkitt Lymphoma), A431 (epidermoid carcinoma), OVCAR-3 (ovarian carcinoma), WiDr, HT-29 (both colon carcinoma), A549 (lung carcinoma), Sk-rc-52 (renal cell carcinoma), PC-3M (prostate carcinoma), A172 (glioblastoma multiforme), HS-683 (medullablastoma). Jurkat.EGFRvIII was generated as previously described (10). RC21 cells (renal cell carcinoma) were generously provided by Prof. Dr. Clemens Löwik (University Medical Center Leiden, Leiden, The Netherlands). All cell lines were cultured at 37°C, in a humidified 5% CO2 atmosphere. All cell lines were cultured in RPMI 1640 (Cambrex Bio Science, Verviers, France) supplemented with 10% fetal calf serum.

Expression of EGFR and TRAIL receptors

Membrane expression levels of EGFR were analyzed using mAb425. Membrane expression levels of TRAIL-receptors 1, 2, 3, and 4, were analyzed by flow cytometry using a TRAIL-receptor antibody kit purchased from Alexis (10P's, Breda, The Netherlands). Briefly, cells were harvested, washed using serum free RPMI, and resuspended in 100 µl fresh medium containing the appropriate primary mAb. Specific binding of the primary antibody was detected using a PE-conjugated secondary antibody (DAKO, Glostrup, Denmark). All antibody incubations were performed at 0°C for 45 min and were followed by two washes with serum free medium.

Recombinant sTRAIL, monoclonal antibodies and inhibitors

Flag-tagged sTRAIL and secondarily cross-linked kTRAIL were both purchased from Alexis. MAb 425 (kindly provided by Merck, Darmstadt, Germany) is a murine IgG2a with high binding affinity for the extra-cellular domain of EGFR and EGFRvIII. TRAIL neutralizing mAb 2E5 was purchased from Alexis (10P's, Breda, The Netherlands). MAb 425 competes with scFv425 for binding to the same epitope. The histone deacetylase inhibitor valproic acid (VPA) was from Sigma-Aldrich (Zwijndrecht, The Netherlands) and was dissolved at 100 mM in dH2O. The cytostatic drug cisplatin was dissolved at 1 mg/ml in 0.9% NaCl. IKK inhibitor wedelolactone was purchased from Sigma-Aldrich and dissolved at 5 mM in DMSO. Caspase inhibitors zIETD-FMK, zLEHD-FMK and zVAD-FMK were purchased

from Calbiochem (VWR International B.V., Amsterdam, The Netherlands) and dissolved at 10 mM in DMSO. Final working concentrations of cisplatin, VPA, caspase inhibitors and wedelolactone were diluted in standard medium.

Production of scFv:sTRAIL fusion proteins

scFv425:sTRAIL-wt, scFv425:sTRAILmR1-5 and MOCK-scFv:sTRAILmR1-5, targeted at the B-cell marker CD20, were constructed and produced essentially as described previously using expression vector pEE14 (10). This plasmid encodes an N-terminal hemagglutinin (HA) tag upstream of two multiple cloning sites. In the first MCS, the high-affinity antibody fragment scFv425 (Vh-(G4S)3-Vl format) (26) was directionally inserted using the unique SfiI and NotI restriction enzyme sites. Alternatively, the upstream MCS of pEE14 was used to insert DNA fragment scFvCD20. The synthetic DNA sequence encoding scFvCD20 was generated by splice overhang extension PCR-technology using published sequence data of the VH - and VL domains of the murine anti-CD20 mAb 2B8. The VH and VL sequences were genetically linked via a flexible peptide linker ((GGGGS)3). Moreover, restriction enzyme sites SfiI (GGCCCAGCCGG) and NotI (GCGGCCGC) were added to the 5'-end and 3'-end of the sequence, yielding a 756 bp DNA fragment. In the second MCS, a PCR-truncated 593 bp DNA fragment encoding the extracellular domain of human TRAIL (sTRAIL) was cloned in frame using restriction enzymes XhoI and HindIII, yielding plasmid pEE14-scFv425:sTRAIL-wt. Alternatively, the cDNA encoding sTRAILmR1-5 was inserted in the second MCS. This sTRAIL mutant encodes 5 amino acid substitutions compared to sTRAIL-wt (25) (see table 1). The resultant expression vectors were transfected into CHO-K1 cells using Fugene 6 reagent (Roche Diagnostics, Almere, The Netherlands) according to manufacturer's instructions. Transfectants were selected by the glutamine synthetase system as described (27). Single cell sorting using the MoFlo high speed cell sorter (Cytomation, Fort Collins, USA) established clones of scFv425:sTRAIL-wt, stably secreting 2.1 µg/ml, scFv425:sTRAILmR1-5, stably secreting 2.7 µg/ml and MOCK-scFv:sTRAILmR1-5, stably secreting 2.3 µg/ml.

Table 1. Changes in amino acid sequence of the sTRAIL domains of scFv425:sTRAILmR1-5 compared to scFv425:sTRAIL-wt.

amino acid pos.	189	191	193	199	201	213	215	264	266	267
scFv425:sTRAIL-wt	Tyr	Arg	Gln	Asn	Lys	Tyr	Ser	His	Ile	Asp
scFv425:sTRAIL-mR1	Tyr	Arg	**Ser**	**Val**	**Arg**	**Trp**	**Asp**	His	Ile	Asp

TRAIL-receptor selective ELISA

TRAIL-receptor binding selectivity of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 was investigated with a catching-type ELISA with either TRAIL-R1-Fc or TRAIL-R2-Fc (both

from Alexis, 10P's BVBA) coated to the plates. Briefly, maxisorb ELISA plates were coated overnight with 1µg/ml TRAIL-R1:Fc or TRAIL-R2:Fc, blocked with PBS/0.1% TWEEN/3% Bovine Serum Albumine (Sigma), washed twice with PBS/0.1% TWEEN, and incubated for 3 hours with scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5, respectively. Specific binding was assessed by staining for the N-terminal hemagglutinin tag using Horseradish Peroxidase-conjugated anti-HA antibody (Roche). Specific binding was visualized using 3,3′,5,5′-Tetramethylbenzidine (TMB)(Sigma) and measured using an ELISA plate reader at OD450. Where indicated, incubation with scFv425:sTRAIL-wt/scFv425:sTRAILmR1-5 was performed in the presence of soluble TRAIL-R1:Fc, TRAIL-R2:Fc or Flag-tagged sTRAIL.

EGFR-specific binding of scFv425:sTRAIL fusion proteins

EGFR-specific binding of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 was assessed by flow cytometry using the EGFR-positive tumor cell line Jurkat.EGFRvIII. In short, $1x10^6$ cells were incubated with fusion protein (300 ng/ml). Specific binding was detected using PE-conjugated anti-TRAIL mAb B-S23 (Diaclone SAS, Besançon, France) and subsequent FACS analysis using a Calibur flow cytometre (Beckman Coulter, Mijdrecht, The Netherlands). Incubations were carried out for 45 min at 0°C and were followed by two washes with serum free medium.

EGFR-restricted induction of apoptosis by scFv425:sTRAIL fusion proteins

EGFR-restricted induction of apoptosis by the scFv425:sTRAIL fusion proteins was assessed by loss of mitochondrial membrane potential ($\Delta\psi$) or by crystal violet cytotoxicity assay as described in more detail below. Where indicated, treatment with scFv425:sTRAIL fusion proteins or the MOCK-scFv:sTRAILmR1-5 was performed in the presence or absence of mAb 425 (3 µg/ml) or mAb 2E5 (1 µg/ml). Loss of mitochondrial membrane potential ($\Delta\psi$); $\Delta\psi$ was analyzed using the stain DiOC6 (Eugene, The Netherlands) as previously described (10). Briefly, cells were pre-cultured in a 48-well plate at a concentration of $0.3x10^5$ cells/well. Subsequently, cells were treated for 16 h with the various experimental conditions, after which cells were harvested and incubated for 20 min with DiOC6 (0,1 µM) at 37°C, harvested (1000g, 5 min.), resuspended in PBS, and assessed for staining by flow cytometry. Cell viability assessed by crystal violet cytotoxicity assay; cells were pre-cultured in a 96-well plate at a concentration of $0.3x10^5$ cells/well. Subsequently, cells were treated for 16 h with the various experimental conditions in a final volume of 200 µl. Cell viability was determined by crystal violet staining (Sigma, Germany) as described previously (8). Experimental apoptosis induction was quantified as the percentage of apoptosis induction compared to medium control. Each experimental condition consisted of six independent wells. Luminescent assay for caspase-8, caspase-9 and caspase-3/-7 activity; caspase activity was assessed using Caspase-Glo® 8 Assay, Caspase-Glo® 9 Assay

and Caspase-Glo® 3/7 Assay according to manufacturer's instructions (Promega Benelux BV, Leiden, The Netherlands). The assays are based on the cleavage of non-luminescent substrates by activated caspases into a luminescent product. Luminescence is quantified using an ELISA plate reader.

Immunoblot analysis of caspase-8, cFLIP$_L$ and NFκB

Cells were seeded in 6 well plates at a final concentration of 2.0x10^6 cells/ml and treated as indicated. Cell lysates were prepared and immunoblot analysis was performed essentially as described before (26). Antibodies used were anti-caspase-8 (Cell signaling technology, Beverly, MA, USA), anti-cFLIP$_L$ clone NF6 (Alexis), NFkB p100/p52, NFkB p105/p50, NFkB p65 (all from Santa-Cruz; Tebu-Bio, Heerhugowaard, The Netherlands). Appropriate secondary PO-conjugated antibodies were from DAKO Cytomation (Glostrup, Denmark).

Selective knock-down of TRAIL-R1 or TRAIL-R2 using small inhibitory (si)RNA

OVCAR-3 cells were pre-cultured in 6-well plates to 60% confluency, after which cells were treated with 10pM TRAIL-R1 siRNA (sense: 5'-CACCAAUGCUUCCAACAAU-3'; antisense: 5'-AUUGUUGGAAGCAUUGGU-3') or TRAIL-R2 siRNA (sense:5'-GACCCUUGUGCUCGUUGUC-3'; antisense: 5'-GACAACGAGCACAAGGGUC-3') (Eurogentec S.A., Liege, Belgium). Treated cells were cultured for 3 days, after which selective TRAIL-receptor down-regulation was verified by flow cytometry. Subsequently, cells were plated in a 48-well plate at 3x10^4 cells/well and treated with scFv425:sTRAIL-wt, scFv425:sTRAILmR1-5, agonistic TRAIL-R1 mAb or agonistic TRAIL-R2 mAb. Apoptosis was assessed by $\Delta\psi$. For siRNA apoptosis experiments, the experimental apoptosis was calculated using the following formula: experimental apoptosis = (specific apoptosis - spontaneous apoptosis)/(100 - spontaneous apoptosis) x 100%.

Synergistic induction of apoptosis by scFv425:sTRAIL fusion proteins and conventional and experimental therapeutic drugs

Cells were plated at 3.0x10^4 cells/well in a 48-well plate and allowed to adhere overnight. Subsequently, cells were concurrently treated for 24h with scFv425:sTRAIL-wt or scFv425:sTRAILmR1-5 with or without cisplatin or histone deacetylase inhibitor VPA. Additive or synergistic apoptotic effects were determined using the cooperativity index (CI). CI was determined with the following formula; the sum of apoptosis induced by single-agent treatment divided by apoptosis induced by combination-treatment. When CI<0.9, treatment was termed synergistic; when 0.9<CI<1.1, treatment was termed additive; when CI>1.1, treatment was termed antagonistic.

IL-8 ELISA

To determine IL-8 production in response to scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5, RC21, PC3-M, HT-29, A549 and WiDr cells were treated with 850 ng/ml fusion protein in the presence or absence of total caspase inhibitor zVAD-FMK. After 16 h, supernatants were analyzed for IL-8 levels using IL-8 ELISA according to manufacturer's protocol (Sanquin reagents, Amsterdam, The Netherlands).

Statistical analysis

Data reported are mean values + standard error of the mean of at least three independent experiments. Where appropriate, statistical analysis was performed using two-sided, unpaired Student' s T-test. For all statistical analyses, a statistically significant difference was defined as $p<0.05$.

Results

EGFR-selective binding and induction of apoptosis by scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5

To determine whether the sTRAILmR1-5 domain of scFv425:sTRAILmR1-5 had any influence on EGFR-specific binding compared to scFv425:sTRAIL-wt, Jurkat.EGFRvIII cells were incubated with scFv425:sTRAIL-wt and scFv425:sTRAIL-mR1 and assessed for EGFR-specific binding (Fig.1A). As expected, both fusion proteins possessed identical binding characteristics to Jurkat.EGFRvIII (Fig. 1A). Binding was EGFR-specific since preincubation with parental EGFR-blocking mAb 425 specifically inhibited the binding of both scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 (data not shown).

To determine whether scFv425:sTRAILmR1-5, similar to scFv425:sTRAIL-wt, showed EGFR-restricted apoptotic activity, EGFR-positive RC21 cells were treated with 350ng/ml scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5. Both fusion proteins potently induced apoptosis in RC21 cells (Fig. 1B). Importantly, induction of apoptosis by scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 in RC21 cells was specifically inhibited by co-incubation with molar excess of parental EGFR-blocking mAb 425 (Fig. 1B). Thus, scFv425:sTRAILmR1-5 selectively binds to EGFR, after which apoptosis is induced by TRAIL-receptor cross-linking.

Subsequently, we determined whether the EGFR-selective accretion of sTRAILmR1-5 to the cell surface of tumor cells resulted in enhanced apoptotic activity compared to non-targeted sTRAILmR1-5. To this end, EGFR-positive cells were treated with scFv425:sTRAILmR1-5 and a MOCK-scFv:sTRAILmR1-5 fusion protein containing an scFv antibody fragment of irrelevant specificity, targeted at the B-cell marker CD20. Dose escalation experiments, exemplified here for PC-3M (Fig. 1C), identified that scFv425:sTRAILmR1-5 showed superior apoptotic activity compared to MOCK-scFv:sTRAILmR1-5 in PC-3M, A431, and RC21 cells (Fig. 1D). Thus, EGFR-selective binding results in superior apop-

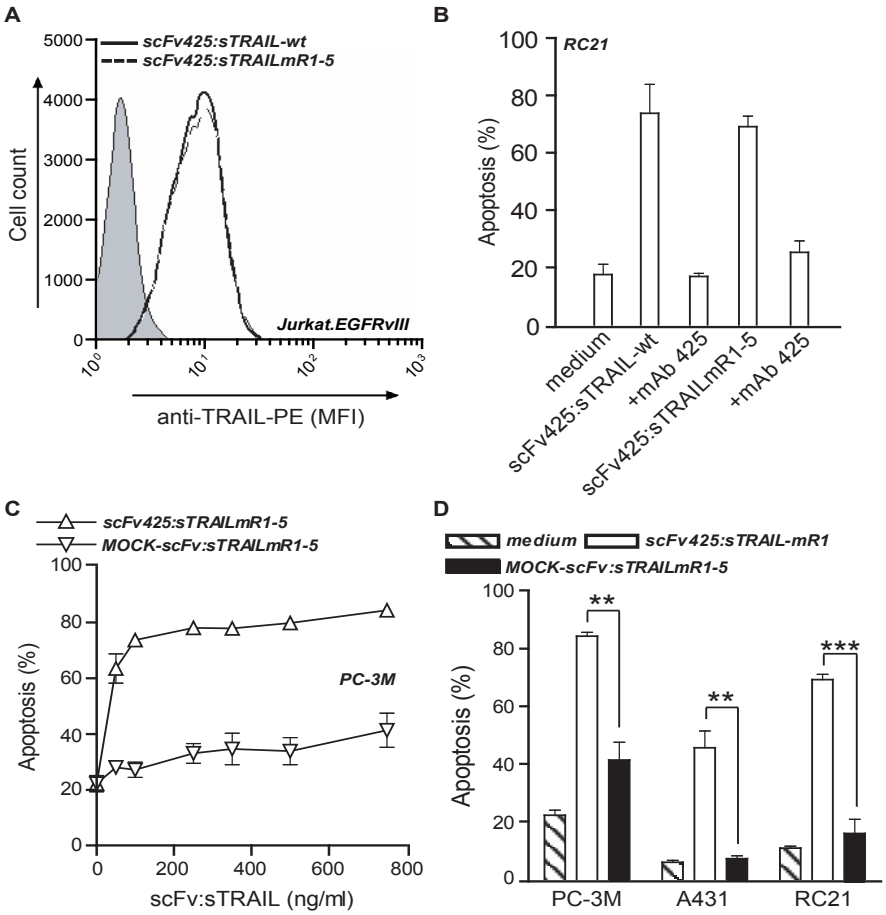

Figure 1. EGFR-selective binding and induction of apoptosis by scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5. A. Jurkat.EGFRvIII cells were incubated with PE-conjugated anti-TRAIL mAb B-S23 (solid fill), scFv425:sTRAIL-wt + mAb B-S23 (solid line), or scFv425:sTRAILmR1-5 + mAb B-S23 (dotted line), after which specific binding was assessed by flow cytometry. **B.** EGFR-positive RC21 cells were treated with 350ng/ml scFv425:sTRAIL-wt or scFv425:sTRAILmR1-5 in the absence or presence of parental EGFR-blocking mAb 425. **C.** PC-3M cells were treated for 16h with increasing concentrations of scFv425:sTRAILmR1-5 or the non-targeted MOCK-scFv:sTRAILmR1-5. D. EGFR-positive cell lines PC-3M, A431 and RC21 were treated with 850 ng/ml scFv425:sTRAILmR1-5 or MOCK-scFv:sTRAILmR1-5. Apoptosis was assessed by $\Delta\psi$. **, $p<0.001$; ***, $p<0.0001$.

totic activity of scFv425:sTRAILmR1-5 compared to the non-targeted soluble MOCK-scFv:sTRAILmR1-5.

scFv425:sTRAILmR1-5 has superior apoptotic activity on a subset of EGFR-positive solid tumor cell lines

To evaluate the apoptotic activity of scFv425:sTRAILmR1-5, we tested a panel of ten EGFR-positive tumor cell lines with scFv425:sTRAILmR1-5 and scFv425:sTRAIL-wt. Dose-escalation experiments, exemplified here with PC-3M cells (Fig. 2A), identified that scFv425:sTRAILmR1-5 had superior apoptotic activity compared to scFv425:sTRAIL-wt in OVCAR3, A549, HT-29, HS-683, A431 and PC-3M cells (Fig. 2A and B). Dose escalation experiments, exemplified here for RC21 (Fig. 2C), on RC21, WiDr, Sk-rc-52, and A172 identified no significant difference in apoptotic activity (Fig. 2D). Of note, in the latter cell lines scFv425:sTRAILmR1-5 consistently showed a non-significant trend towards higher apoptotic activity compared to scFv425:sTRAIL-wt.

To determine whether scFv425:sTRAILmR1-5 retained its superior apoptotic activity compared to scFv425:sTRAIL-wt upon co-treatment with other conventional/experimental anti-cancer therapeutics, OVCAR-3 cells were treated with the respective fusion proteins alone or in combination with VPA or cisplatin (Fig. 3A and B). Importantly, co-treatment with cisplatin and VPA synergistically enhanced the apoptotic activity of both scFv425:sTRAIL-wt (CI of 0.73 and 0.42, respectively) and scFv425:sTRAILmR1-5 (CI of 0.57 and 0.42, respectively). However, the scFv425:sTRAILmR1-5 fusion protein retained a significantly higher pro-apoptotic activity compared to scFv425:sTRAIL-wt ($p<0.001$ and $p<0.05$, respectively). The synergistic effect of co-treatment with VPA and cisplatin was still fully dependent on EGFR-selective binding of the respective fusion protein, since co-treatment with EGFR-blocking mAb 425 abrogated the induction of apoptosis (data not shown).

The apoptotic activity of scF425:sTRAIL-wt and scFv425:sTRAILmR1-5 does not correlate with EGFR- or TRAIL-receptor expression

Since the sTRAILmR1-5 domain was described to be selective for TRAIL-R1, we subsequently analyzed whether the differences in apoptotic activity of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 in the subset of cell lines was due to differential TRAIL-receptor expression. To this end, we determined the relative TRAIL-receptor expression levels of the cell lines as well as the expression level of EGFR (Table 2). In agreement with our previous findings for scFv425:sTRAIL-wt, the activity of scFv425:sTRAILmR1-5 did not correlate with the level of EGFR expression. Importantly, there was also no correlation with the expression levels of TRAIL-R1, TRAIL-R2 or TRAIL-R4 and the apoptotic activity of the fusion proteins. In addition, there was not a clear correlation between the various ratios of TRAILR2/TRAIL-R1, TRAIL-R1/TRAIL-R4 or TRAIL-R2/TRAIL-R4, although 4 out of the 6

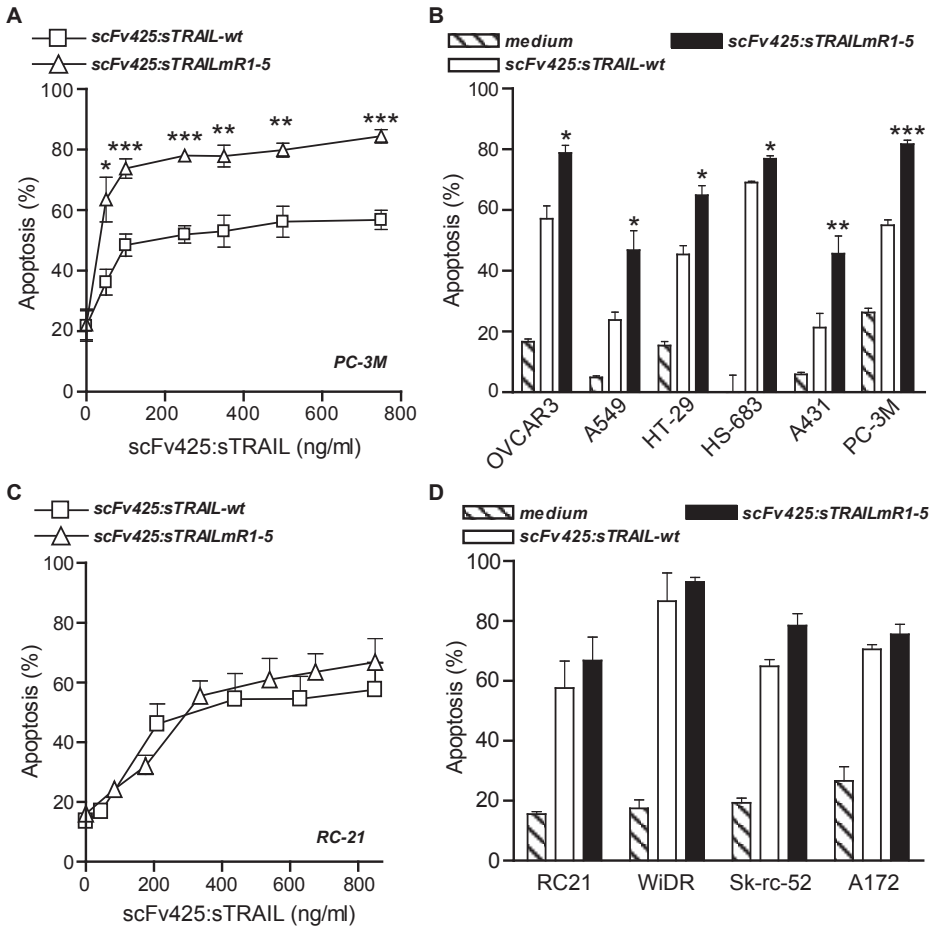

Figure 2. Fusion protein scFv425:sTRAILmR1-5 has superior apoptotic activity on a subset of EGFR-positive solid tumor cell lines. A. Dose-response curve of induction of apoptosis in PC-3M cells by increasing concentrations of scFv425:sTRAIL-wt or scFv425:sTRAILmR1-5. **B.** OVCAR3, A549, HT-29, HS-683, A431 and PC-3M cells were treated with scFv425:sTRAIL-wt (850 ng/ml) or scFv425:sTRAILmR1-5 (850 ng/ml). Cell viability in HS-683 was assessed by crystal violet toxicity assay. **C.** Dose-response curve of apoptosis induction in RC21 cells treated with increasing concentrations of scFv425:sTRAIL-wt or scFv425:sTRAILmR1-5. **D.** RC21, WiDr, Sk-rc-52 and A172 cells were treated with scFv425:sTRAIL-wt (850 ng/ml) or scFv425:sTRAILmR1-5 (850 ng/ml). Unless indicated otherwise, apoptosis was assessed by $\Delta\psi$. *, $p<0.05$; **, $p<0.001$; ***, $p<0.0001$.

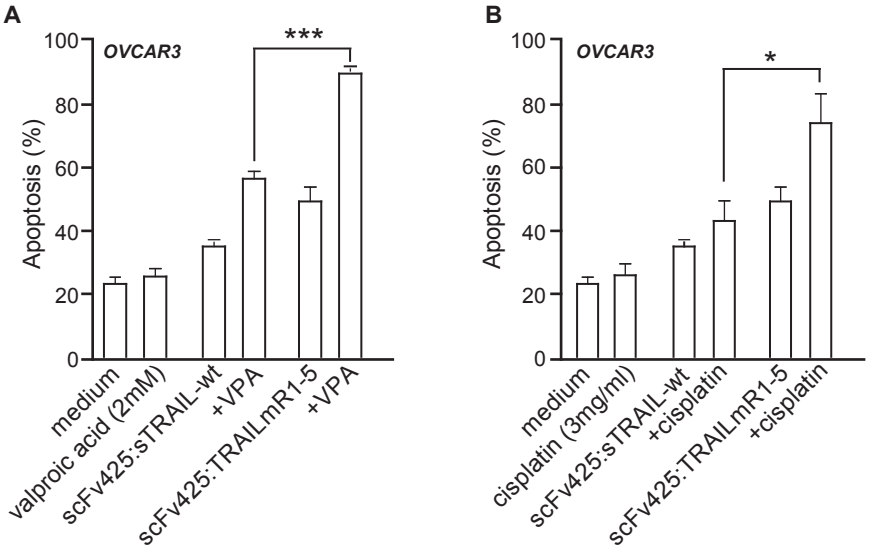

Figure 3. Fusion protein scFv425:sTRAILmR1-5 has superior activity in combination with conventional/experimental therapeutics. A. OVCAR3 cells were treated alone or with a combination of the HDACi VPA (2 mM) and scFv425:sTRAIL-wt (350 ng/ml) or scFv425:sTRAILmR1-5 (350ng/ml). **B.** OVCAR3 cells were treated alone or with a combination of cisplatin (3 µg/ml) and scFv425:sTRAIL-wt (350 ng/ml) or scFv425:sTRAILmR1-5 (350 ng/ml). Unless indicated otherwise, apoptosis was assessed by $\Delta\psi$. *, $p<0.05$; **, $p<0.001$; ***, $p<0.0001$.

(66%) cell lines that were significantly more sensitive to scFv425:sTRAILmR1-5 appeared to have a more balanced TRAIL-R2/TRAIL-R1 ratio, in comparison to 2 out of 4 (50%) of the other cell lines. A particularly intriguing finding is the fact that some of the cell lines, most sensitive to scFv425:sTRAILmR1-5, have a very low expression of TRAIL-R1 (Table 2, HT29; MFI of 8.1, HS683; MFI of 0.5, PC-3M; MFI of 1.9).

scFv425:sTRAIL-wt but also scFv425:sTRAILmR1-5 binds to both TRAIL-R1 and TRAIL-R2

The sTRAILmR1-5 mutant we genetically fused to scFv425 was reported by MacFarlane et al. to selectively activate TRAIL-R1. However, we found no clear correlation between the TRAIL-R1 status and activity of scFv425:TRAILmR1-5, with in certain cell lines very low TRAIL-R1 expression that coincided with high apoptotic activity of scFv425:sTRAILmR1-5. Therefore, we performed a catching-type ELISA in which plates were coated either with TRAIL-R1-Fc or TRAIL-R2-Fc to assess whether the sTRAILmR1-5 domain was indeed selective for TRAIL-R1. As expected, binding of scFv425:sTRAIL-wt was observed in both TRAIL-R1:Fc and TRAIL-R2:Fc coated plates (Fig. 4A and B, open squares). Surprisingly

Table 2. EGFR/TRAIL-receptor expression and the correlation with the apoptotic activity of scFv425:sTRAIL-wt/scFv425:sTRAILmR1-5. The percentage of induction of apoptosis by scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 are the percentages obtained by treatment with 850 ng/ml. P-values were obtained using unpaired Student's T-test. The MFI values of EGFR and TRAIL-receptor expression are representatives of three independent experiments. n.s.; no significant difference, n.d.; not determined

Cell line	scFv425:sTRAIL-wt	scFv425:sTRAILmR1-5	P-value	EGFR	TRAIL-R1	TRAIL-R2	TR2/TR1	TRAIL-R4	TR1/TR4	TR2/TR4	p53 status
OVCAR-3	57,1±8,6	78,8±5,0	0,0048	629	167,1	207,8	1,2	n.a.	-	-	mut
A549	23,8±5,7	46,8±14,4	0,0106	1.755	69,7	151,5	2,2	8,3	8,4	18,2	wt
HT-29	51,5±1,7	65,2±4,5	0,0468	317	8,1	35,1	4,3	5,2	1,6	6,8	mut
HS683	69±0,9	76,87±2,1	0,0313	436	0,5	44,8	98,4	8,6	0,1	5,2	mut
A431	21,3±8,1	45,6±10,1	0,0313	2727	4,7	7,8	1,7	n.a.	-	-	mut
PC3-M	56,8±3,2	84,4±2,2	0,0003	34	1,9	45,3	23,4	6,86	0,3	6,6	null
RC21	57,6±18,0	66,8±13,6	n.s.	3.648	214,9	398,6	1,9	29,6	7,3	13,5	n.a.
Sk-rc-52	64,9±4,6	78,49±6,9	n.s.	1.263	12,1	133,0	11,0	13,5	0,9	9,9	n.a.
A172	70,5±2,3	75,5±4,8	n.s.	182	0,6	112,2	178,2	3,6	0,2	31,2	wt
WiDR	86,6±13,3	93±2,2	n.s.	256	17,8	56,3	3,2	1,9	9,5	29,9	mut

however, scFv425:sTRAILmR1-5 also proved to bind to both TRAIL-R1:Fc and TRAIL-R2:Fc (Fig. 4A and B, open triangles).

To establish whether this interaction was indeed specific, we competitively inhibited the binding of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5, as observed in the TRAIL-R1:Fc ELISA, using soluble TRAIL-R1:Fc and TRAIL-R2:Fc (Fig 4C and D). Importantly, binding of both fusion proteins was competitively inhibited by TRAIL-R1:Fc and TRAIL-R2:Fc (Fig. 4C and D). Of note, whereas scFv425:sTRAIL-wt binding was more efficiently inhibited by addition of TRAIL-R2:Fc, binding of scFv425:sTRAIL-mR1-5 was more efficiently inhibited by TRAIL-R1:Fc. Thus, scFv425:sTRAILmR1-5 appears to preferentially bind to TRAIL-R1, whereas scFv425:sTRAIL-wt appears to preferentially bind to TRAIL-R2. In addition, binding of scFv425:sTRAIL-wt and scFv425:sTRAIL-mR1-5 to TRAIL-R1:Fc was competitively inhibited by Flag-tagged wildtype sTRAIL (Fig. 4E). Together, these results clearly show that scFv425:sTRAILmR1-5 specifically binds to not only TRAIL-R1 but also TRAIL-R2.

scFv425:sTRAIL-wt but also scFv425:sTRAILmR1-5 activate apoptosis via both TRAIL-R1 and TRAIL-R2

To determine whether binding of scFv425:sTRAILmR1-5 to TRAIL-R2 also had functional consequences, RC21 renal cancer cells (TRAIL-R1$^+$/R2$^+$) were treated with scFv425:sTRAILmR1-5 in the presence of TRAIL-R1 blocking mAb, TRAIL-R2 blocking mAb, or a combination of both mAbs. Importantly, induction of apoptosis by scFv425:sTRAILmR1-5 in RC21 cells was significantly inhibited not only by TRAIL-R1 mAb, but also by the TRAIL-R2 mAb (Fig. 5A). Of note, the TRAIL-R2 mAb was even more effective than the TRAIL-R1 mAb in blocking scFv425:sTRAILmR1-5 activity. Co-treatment with both blocking mAbs abrogated the apoptotic activity of scFv425:sTRAILmR1-5. Similar results were obtained for scFv425:sTRAIL-wt (Fig.5B) as well as killer TRAIL (kTRAIL) (Fig.5C), an artificially

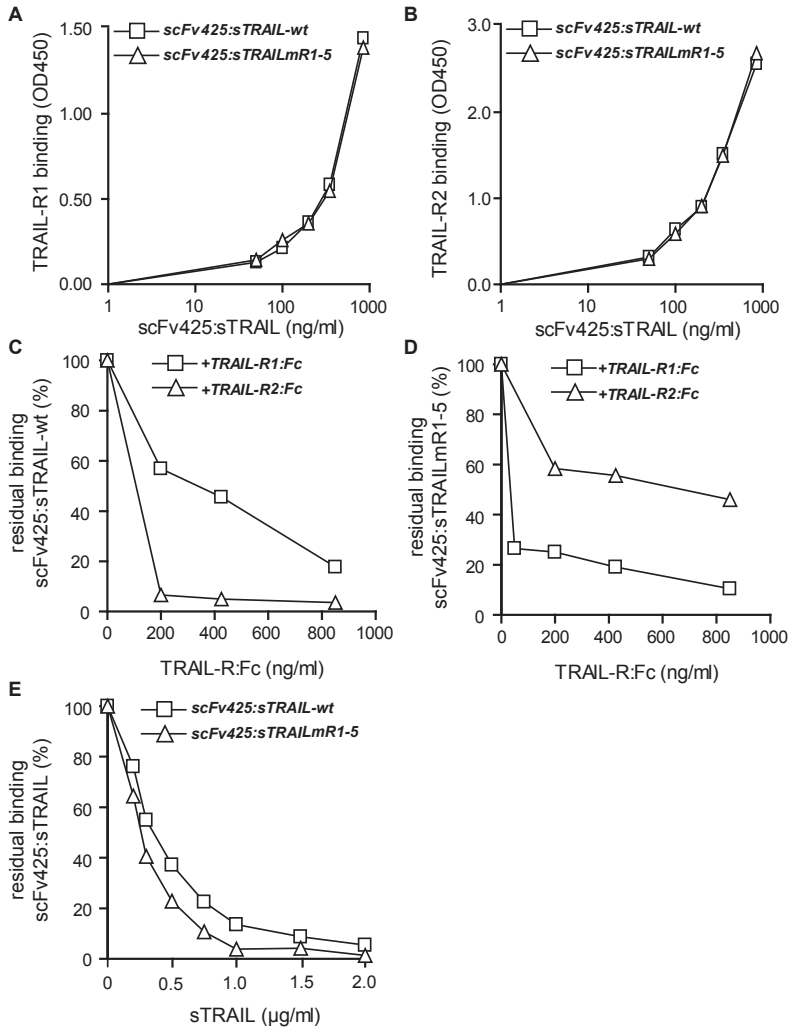

Figure 4. scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 bind to TRAIL-R1 and TRAIL-R2. A. Increasing concentrations of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 were incubated with TRAIL-R1:Fc coated plates. **B.** Increasing concentrations of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 were incubated with TRAIL-R2:Fc coated plates. **C.** Binding of scFv425:sTRAIL-wt (850 ng/ml) was competitively inhibited by co-incubation with increasing concentrations of TRAIL-R1:Fc and TRAIL-R2:Fc. **D.** Binding of scFv425:sTRAILmR1-5 (850 ng/ml) was competitively inhibited by co-incubation with increasing concentrations of TRAIL-R1:Fc and TRAIL-R2:Fc. **E.** Binding of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 (600 ng/ml) was competitively inhibited by co-incubation with increasing concentrations of recombinant Flag-tagged sTRAIL-wt. In all experiments, TRAIL-receptor specific binding was determined by spectrophotometry at OD450 as described in materials and methods.

cross-linked sTRAIL preparation. However, compared to the scFv425:sTRAILmR1-5 fusion protein, both scFv425:sTRAIL-wt as well as kTRAIL were far less efficiently blocked by TRAIL-R1 mAb, TRAIL-R2 mAb or the combination of both mAbs.
In addition, treatment of RC21 cells with scFv425:sTRAILmR1-5 in the presence of TRAIL-R1:Fc or TRAIL-R2:Fc potently inhibited the induction of apoptosis (Fig. 5D). Of note, TRAIL-R2:Fc was more efficient than TRAIL-R1:Fc in blocking apoptosis. Similarly, TRAIL-R1:Fc and TRAIL-R2:Fc also inhibited the apoptotic activity of scFv425:sTRAIL-wt and kTRAIL (Fig. 5D), although again to a lesser extent than scFv425:sTRAILmR1-5.
To further verify that both TRAIL-R1 and TRAIL-R2 signaling are involved in activation of apoptosis in solid tumor cells by scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5, siRNA-mediated knock-down of TRAIL-R1 or TRAIL-R2 was performed in OVCAR3 cells. Normally, OVCAR3 cells express TRAIL-R1 and TRAIL-R2 at approximately equal levels on the cell surface (Fig. 6A). In addition, treatment with scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 potently activates apoptosis (Fig. 6B). Treatment of OVCAR3 cells with TRAIL-R1 specific siRNA resulted in ~92% down-regulation of TRAIL-R1 (Fig. 6C). Similarly, treatment with TRAIL-R2 specific siRNA resulted in ~91% down-regulation of TRAIL-R2 (Fig. 6D).
The absence of TRAIL-R1 or TRAIL-R2 signaling, respectively, upon siRNA treatment was confirmed using TRAIL-receptor selective agonistic antibodies. A TRAIL-R1 agonistic mAb was largely inactive on OVCAR3 cells treated with TRAIL-R1 siRNA (Fig. 7A), while a TRAIL-R2 agonistic mAb was largely inactive on OVCAR3 cells treated with TRAIL-R2 siRNA (Fig. 7B). Thus, treatment with the respective TRAIL-R1 or TRAIL-R2 siRNA indeed functionally down-regulates the receptor. However, scFv425:sTRAILmR1-5 potently induced apoptosis in these cells irrespective of TRAIL-R1 or TRAIL-R2 down regulation (Fig. 7A and B), indicating that both receptors can be activated by scFv425:sTRAILmR1-5. In addition, the pro-apoptotic activity of scFv425:sTRAIL-wt was markedly reduced compared to scFv425:sTRAILmR1-5 when TRAIL-R2 was knocked-down, thus confirming previous reports that wild-type sTRAIL preferentially signals via TRAIL-R2.

scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 activate similar caspase-signaling
The differential reliance on TRAIL-R1/R2 for induction of apoptosis by scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 after siRNA-mediated receptor down regulation, prompted us to assess whether the type of apoptosis induced by the two fusion proteins was similar or divergent. Therefore, we treated RC21 and PC-3M cells with scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 and determined caspase-activity at various time-points. In both RC21 and PC-3M cells, induction of apoptosis by scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 was associated with a similar activation profile for caspase-8 (Fig. 8A), caspase-9 (Fig. 8B) and caspase-3/-7 (Fig. 8C). As expected, caspase levels for

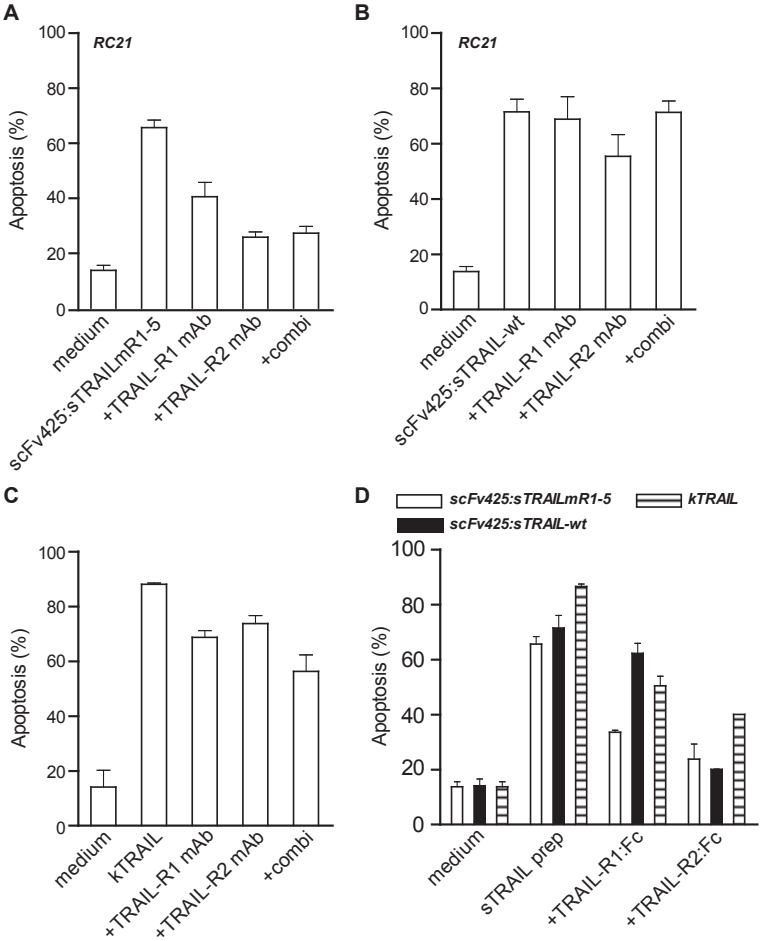

Figure 5. Induction of apoptosis by scFv425:sTRAILmR1-5 and MOCK-scFv:sTRAILmR1-5 is inhibited by TRAIL-R2 blocking mAb. RC21 cells were treated with **A.** scFv425:sTRAILmR1-5 (500 ng/ml), **B.** scFv425:sTRAIL-wt (500 ng/ml) and **C.** kTRAIL (50 ng/ml) in the presence or absence of TRAIL-R1 blocking mAb (1 µg/ml), TRAIL-R2 blocking mAb (1 µg/ml), or a combination of TRAIL-R1 and TRAIL-R2 blocking mAbs. **D.** RC21 cells were treated with scFv425:sTRAILmR1-5 (500 ng/ml), scFv425:sTRAIL-wt (500 ng/ml), or kTRAIL (50 ng/ml) in the presence or absence of TRAIL-R1:Fc or TRAIL-R2:Fc (both 1 µg/ml). In all experiments, apoptosis was assessed by $\Delta\psi$.

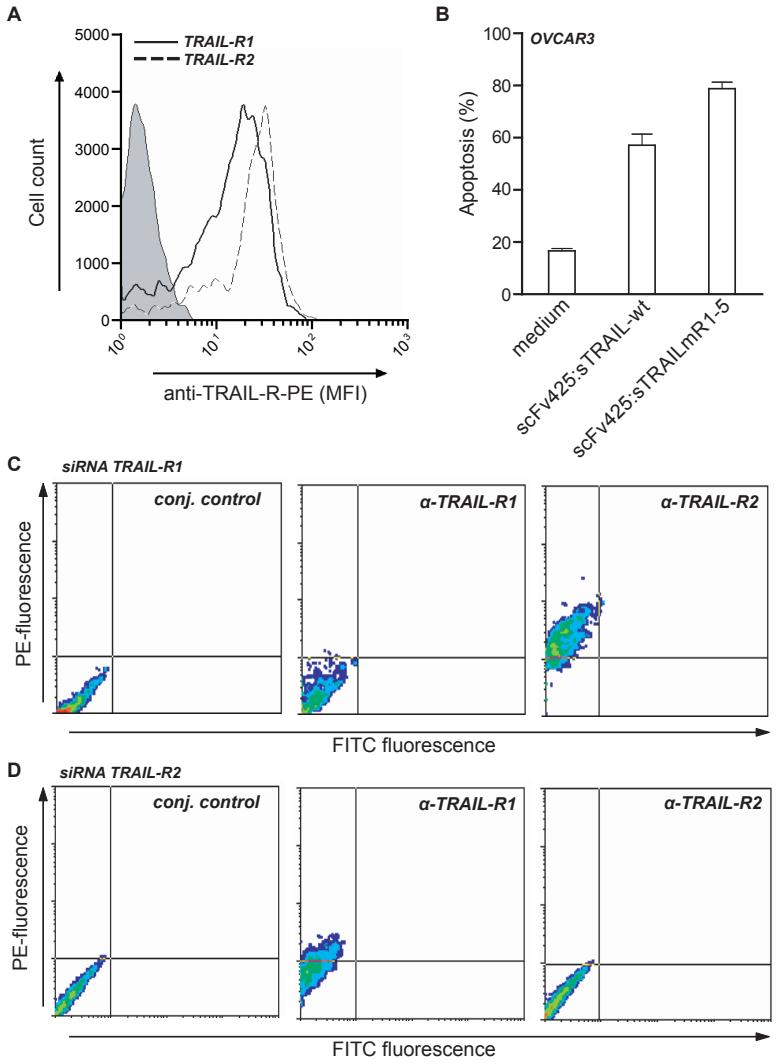

Figure 6. siRNA-mediated down-regulation of TRAIL-R1 or TRAIL-R2. A. OVCAR3 cells were incubated with PE-conjugated Goat-anti-Mouse polyclonal Ab (GaM-PE) (solid fill), TRAIL-R1 mAb + GaM-PE (solid line), or TRAIL-R2 mAb + GaM-PE (dotted line), after which TRAIL-receptor expression was assessed by flow cytometry as described in materials and methods. **B.** OVCAR3 cells were treated with scFv425:sTRAIL-wt (850 ng/ml) or scFv425:sTRAILmR1-5 (850 ng/ml). **C.** OVCAR3 cells were treated for 72h with TRAIL-R1 siRNA and **D.** TRAIL-R2 siRNA, after which expression of TRAIL-receptors was assessed by incubating cells with TRAIL-R1 mAb and TRAIL-R2 mAb. The depicted histograms are representatives of three independent experiments.

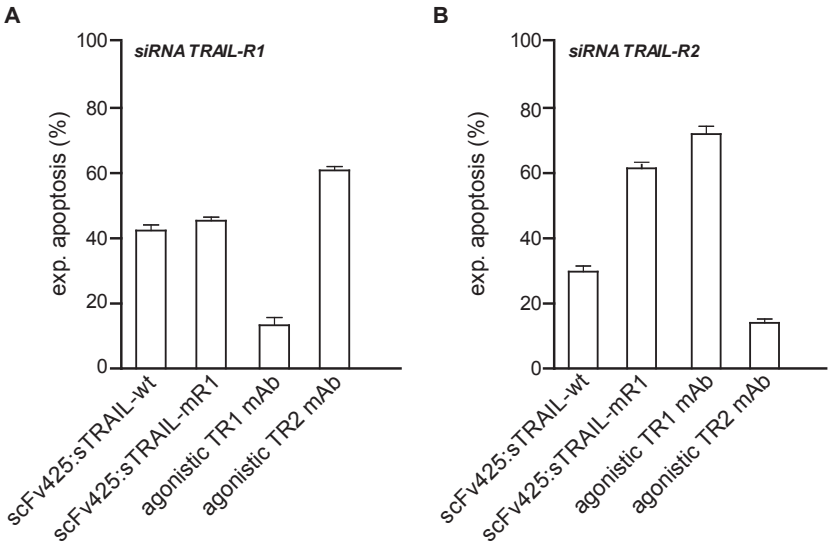

Figure 7. scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 activate apoptosis after siRNA-mediated down-regulation of TRAIL-R1 or TRAIL-R2. A. After incubation with TRAIL-R1 siRNA for 72h, OVCAR3 cells were treated with scFv425:sTRAIL-wt, scFv425:sTRAILmR1-5, agonistic TRAIL-R1 mAb, or agonistic TRAIL-R2 mAb. **B.** After incubation with TRAIL-R2 siRNA for 72h, OVCAR3 cells were treated with scFv425:sTRAIL-wt, scFv425:sTRAILmR1-5, agonistic TRAIL-R1 mAb, or agonistic TRAIL-R2 mAb. In all experiments, apoptosis was assessed by $\Delta\psi$.

scFv425:sTRAILmR1-5 were consistently higher in PC-3M cells (Fig. 8A, B and C). Subsequent inhibition of caspase-8, caspase-9 or total caspases confirmed that scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 have a similar dependency on caspases for execution of apoptosis (Fig. 8D). Interestingly, immunoblot analysis of the caspase-8 inhibitor $cFLIP_L$ revealed a striking difference between scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5. Cells treated with scFv425:sTRAILmR1-5 displayed a total absence of $cFLIP_L$, whereas in scFv425:sTRAIL-wt treated cells $cFLIP_L$ was markedly upregulated compared to medium control (Fig. 8E and F). However, this difference was found for both RC21 and PC-3M cells suggesting that it is not the underlying reason for the differential activity of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5.

The differential activity of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 is not due to NFκB signaling

Analysis of the pro-apoptotic signaling pathways by scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 revealed an intriguing difference in $cFLIP_L$. Since this apoptotic modulator has been linked to the NFκB pathway, we further analyzed the NFκB

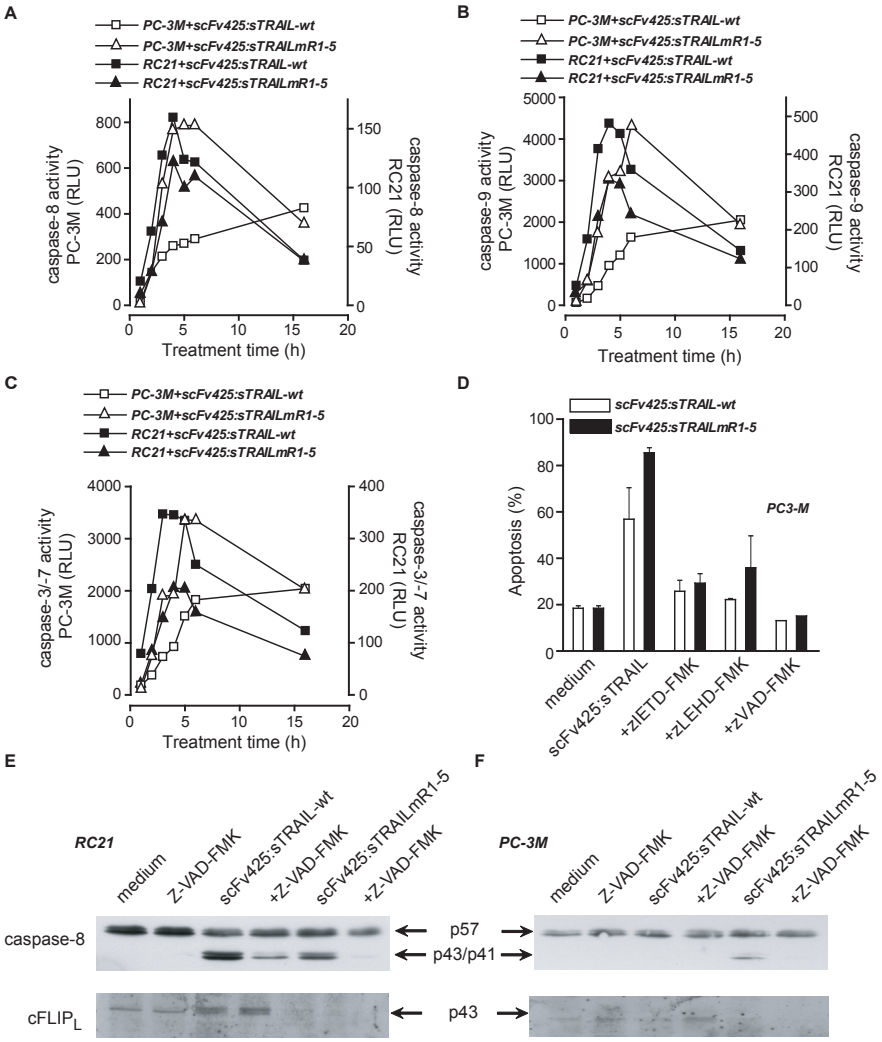

Figure 8. scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 activate similar caspase-signaling. Activity of **A.** Caspase-8, **B.** Caspase-9 and **C.** Caspase-3/-7 was assessed in PC-3M and RC21 cells after incubation for 1, 2, 3, 4, 5, 6 or 16h with either scFv425:sTRAIL-wt (850 ng/ml) or scFv425:sTRAILmR1-5 (850 ng/ml) **D.** PC-3M cells were treated for 16h with scFv425:sTRAIL-wt (850 ng/ml) or scFv425:sTRAILmR1-5 (850 ng/ml) in the presence or absence of zIETD-FMK (20 μM), zLEHD-FMK (20 μM) or zVAD-FMK (20 μM). Apoptosis was assessed by Δψ. **E.** RC21 and **F.** PC-3M cells were incubated for 6h with scFv425:sTRAIL-wt (850 ng/ml) or scFv425:sTRAILmR1-5 (850 ng/ml) in the presence or absence of zVAD-FMK (20 μM). Whole cell lysates were analyzed for the presence of caspase-8 and $cFLIP_L$.

survival signaling as measured by IL-8 production in response to scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 treatment in RC21 and PC-3M cells (Fig. 9A). Contrary to expectations, scFv425:sTRAILmR1-5 consistently induced more NFκB signaling than scFv425:sTRAIL-wt, also in A549, HT-29 and WiDr cells (Fig. 9B). Interestingly, upon immunoblot analysis of p100/p52 and p105/p50 a clear processing of p100 into p52 was detected in scFv425:sTRAIL-wt, but not in scFv425:sTRAILmR1-5 treated cells in both RC21 and PC-3M (Fig. 9C and D), indicating that the fusion proteins activate different subunits of NFκB. To determine the relevance of this observation for the differential apoptotic activity of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5, NFκB-signaling was inhibited using IKK inhibitor Wedelolactone. Unfortunately, co-treatment yielded a similar and marginal increase in apoptosis for both fusion proteins (Fig. 9E and F). Thus, although an intriguing difference in NFκB signaling exists between scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5, this can not account for the differential activity of these fusion proteins on PC-3M.

Discussion

Insight into the contribution of the respective agonistic TRAIL-receptors to induction of apoptosis by sTRAIL in selected tumor types may help optimize TRAIL-based therapeutic strategies. The role of TRAIL-R1 in TRAIL-signaling in particular is subject to debate. Therefore, we here evaluated the pro-apoptotic activity of fusion protein scFv425:sTRAILmR1-5, comprising EGFR-blocking antibody fragment scFv425 and the previously described TRAIL-R1 selective sTRAIL mutant sTRAILmR1-5 (25). Fusion protein scFv425:sTRAILmR1-5 showed significantly superior apoptotic activity compared to the corresponding scFv425:sTRAIL-wt fusion protein on ~60% of the EGFR-positive solid tumor cell lines tested and showed a non-significant trend to higher apoptotic activity on the other cell lines. Both scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 showed synergistic pro-apoptotic activity upon co-treatment with cisplatin or the histone deacetylase inhibitor VPA, but scFv425:sTRAILmR1-5 remained significantly more potent when compared to the wild type sTRAIL fusion protein. Induction of apoptosis by scFv425:sTRAILmR1-5 as well as the synergistic induction of apoptosis with VPA and cisplatin is dependent on binding to EGFR, since co-incubation with parental EGFR-blocking mAb 425 strongly inhibits apoptosis. Importantly, compared to a non-targeted MOCK-scFv:sTRAILmR1-5 fusion protein, directed at the B-cell marker CD20, scFv425:sTRAILmR1-5 showed superior apoptotic activity.

Mutant sTRAILmR1-5 was reported by MacFarlane et al. to be selective for TRAIL-R1. This conclusion was mainly based on the differential apoptotic activity of this mutant towards Jurkat (TRAIL-R1$^-$/TRAIL-R2$^+$) and Ramos cells (TRAIL-R1$^+$/TRAIL-R2$^+$) (24). However, our experiments collectively demonstrate that sTRAILmR1-5 is actually not selective for TRAIL-R1. In ELISA experiments scFv425:sTRAILmR1-5 bound to TRAIL-R2:Fc in a

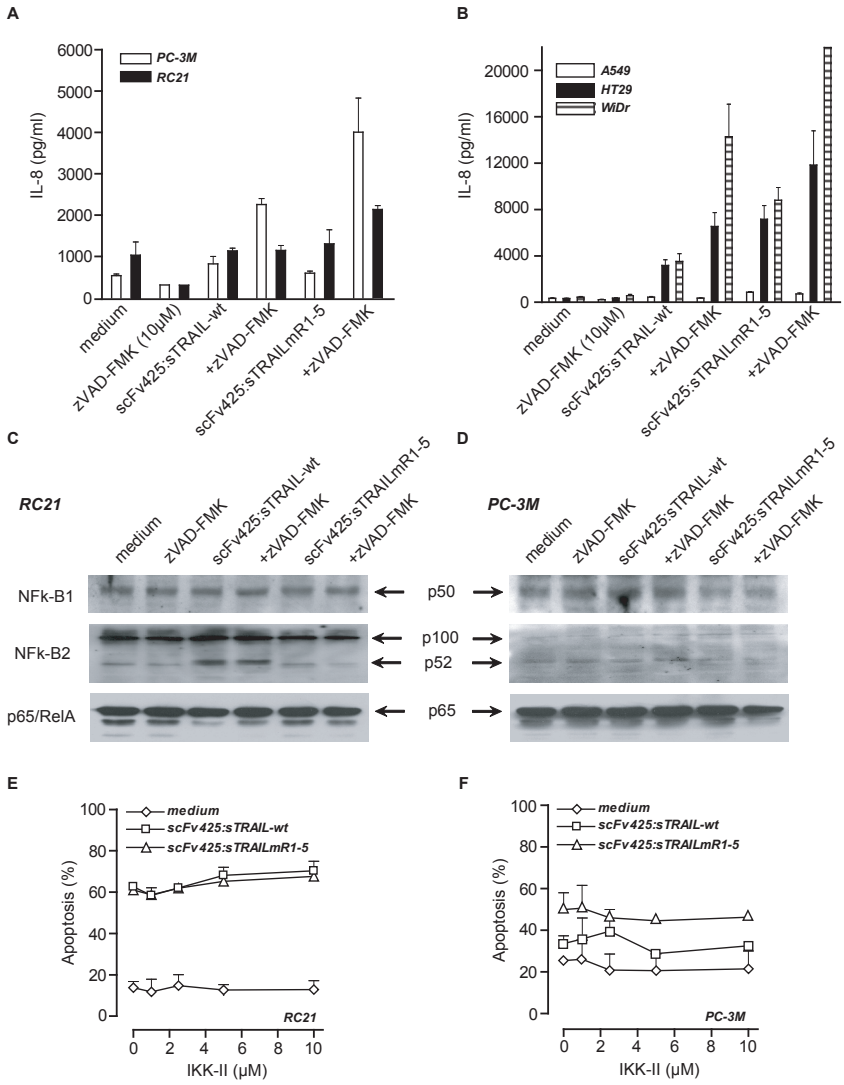

Figure 9. The differential activity of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5 is not due to NFκB signalling. A. RC21, PC-3M and **B.** A549, HT29 and WiDr cells were incubated for 16h with scFv425:sTRAIL-wt (850 ng/ml) or scFv425:sTRAILmR1-5 (850 ng/ml) in the presence or absence of zVAD-FMK (20 μM), after which supernatant was analyzed for IL-8 levels. **C.** RC21 and **D.** PC-3M cells were incubated in the presence or absence of zVAD-FMK (20 μM). Whole cell lysates were analyzed for the presence of NFκB subunits p100/p52, p105/p50 and p65. **E.** RC21 and **F.** PC-3M cells were treated for 6h with scFv425:sTRAIL-wt (850 ng/ml) or scFv425:sTRAILmR1-5 (850 ng/ml) in the presence of IKK inhibitor wedelolactone. Apoptosis was assessed by $\Delta\psi$.

catching-type ELISA. Furthermore, binding of scFv425:sTRAILmR1-5 to TRAIL-R1:Fc was competitively inhibited by addition of soluble TRAIL-R2:Fc. Interestingly, TRAIL-R1:Fc was superior to TRAIL-R2:Fc in inhibiting scFv425:sTRAILmR1-5, whereas the reverse was true for scFv425:sTRAIL-wt, indicating a reversal in affinity between sTRAIL-wt and the sTRAILmR1-5 domain for TRAIL-R1 and TRAIL-R2. Of note, the only interaction that is being investigated in these ELISAs is that of the sTRAIL domain with TRAIL-receptors. Thus, these experiments demonstrate that the sTRAILmR1-5 domain in itself is capable of binding to TRAIL-R2.

The binding of the sTRAILmR1-5 domain to TRAIL-R2 also has functional implications, since induction of apoptosis by scFv425:sTRAILmR1-5 was markedly inhibited by a TRAIL-R2 blocking antibody. In actual fact, the TRAIL-R2 blocking mAb was more effective than the TRAIL-R1 blocking mAb. Moreover, ~92% down-regulation of TRAIL-R1 by small inhibitory RNA did not abrogate the apoptotic activity of scFv425:sTRAILmR1-5. Indeed, some of the more sensitive cell lines actually have very low TRAIL-R1 expression (Table 2). Collectively, these data indicate that target antigen-bound scFv425:sTRAILmR1-5 is capable of binding to and activation of TRAIL-R2. Therefore, our data also warrant a re-evaluation of the conclusion of MacFarlane et al. that non-targeted sTRAILmR1-5 is selective for TRAIL-R1.

Interestingly, induction of apoptosis by scFv425:sTRAILmR1-5 fusion protein was more effectively inhibited by TRAIL-R blocking mAbs than the induction of apoptosis by scFv425:sTRAIL-wt or kTRAIL. These experiments suggest that the wild-type sTRAIL domain has a relatively higher affinity for its receptors and can thus more efficiently compete with the TRAIL-R blocking mAbs for binding to the receptors. Together, this also implies that, while en route to the tumor, scFv425:sTRAILmR1-5 will display a more transient binding to the ubiquitously expressed TRAIL-receptors. As a result, tumor cell accretion is expected to be more efficient for scFv425:sTRAILmR1-5 compared to scFv425:sTRAIL-wt.

Our experimental data regarding the activity of scFv425:sTRAILmR1-5 also highlight an intriguing question, namely: what is the molecular mechanism for the enhanced activity of this sTRAIL mutant on EGFR-positive tumor cell lines as reported here and on primary patient-derived B-CLL cells as reported previously. First and foremost, based on our experimental evidence it is obvious that both TRAIL-R1 and TRAIL-R2 signaling are an integral part of the apoptotic activity of this mutant. However, there is no obvious correlation between the apoptotic activity of scFv425:sTRAILmR1-5 and the expression levels of the respective TRAIL-receptors, nor the ratios of the various TRAIL-receptors (TRAIL-R2/TRAIL-R1, TRAIL-R1/TRAIL-R4, TRAIL-R2/TRAIL-R4).

One possible explanation for our and MacFarlane's observations may be found in the incompletely characterized complexity of TRAIL-receptor biology. Several groups have found experimental evidence that TRAIL-receptors can not only exist in a homotrimeric form, but

can also form heterotrimeric complexes comprising TRAIL-R1 and TRAIL-R2 monomers (28,29). As such, TRAIL-R1/TRAIL-R2 heterotrimers may represent nature's strategy to fine tune the apoptotic response to TRAIL.

It is tempting to speculate that sTRAILmR1-5, besides activating homotrimeric TRAIL-R1, may be better able to activate TRAIL-R1/R2 heterotrimers than wild type sTRAIL. Such a differential capacity to activate heterotrimers might explain the superior induction of apoptosis by scFv425:sTRAILmR1-5 in a subset of cell lines. This hypothesis is in line with most of our observations, including the finding that scFv425:sTRAILmR1-5 is inactive on A2780 cells that only express TRAIL-R2 (data not shown), while induction of apoptosis by scFv425:sTRAILmR1-5 is potently inhibited by a TRAIL-R2 agonistic antibody on TRAIL-$R1^+$/TRAIL-$R2^+$ cells. Moreover, siRNA knock-down of either TRAIL-R1 or TRAIL-R2 by >90% does not block induction of apoptosis by scFv425:sTRAILmR1-5.

Further circumstantial evidence for heterotrimeric TRAIL-R1/TRAIL-R2 can be found in a recent report in which it was shown that selective activation of either TRAIL-R1 or TRAIL-R2 with receptor specific agonistic mAbs in B-CLL cells is not as effective as sTRAIL-mediated apoptosis of the same cells (30). These results point to a benefit of simultaneous triggering of both TRAIL-receptors. Interestingly, sTRAIL versions such as Apo2L/sTRAIL (Genentech) that activate apoptosis predominantly through TRAIL-R2 were less efficacious than those versions that also signal apoptosis via TRAIL-R1, which is in agreement with our finding that scFv425:sTRAILmR1-5 is more active than scFv425:sTRAIL-wt.

An alternative or complementary hypothesis for the enhanced activity of scFv425:sTRAILmR1-5 may be found in the recently described potential for S-nitrosylation of TRAIL-R1 and not TRAIL-R2 (31). Tang et al. found that treatment of OVCAR3 cells with a nitric oxide donor resulted in the S-nitrosylation of TRAIL-R1 at cysteine residue 336, with a concomitant superior induction of apoptosis by TRAIL. Therefore, the presence of S-nitrosylated TRAIL-R1 in certain cell lines, intrinsically subject to higher nitric oxide stress, may account for the superior apoptotic activity of scFv425:sTRAILmR1-5.

Importantly, the superior apoptotic activity of scFv425:sTRAILmR1-5 on certain cell lines is not related to PI3K signaling, JNK-signaling (data not shown), nor on differential caspase-signaling or NFκB-signaling. However, independent of the differential apoptotic activity of scFv425:sTRAIL-wt and scFv425:sTRAILmR1-5, we observed an intriguing difference in the level of $cFLIP_L$ in treated cells. Treatment with scFv425:sTRAIL-wt was associated with an increased level of $cFLIP_L$, whereas scFv425:sTRAILmR1-5 treated cells lacked $cFLIP_L$ expression altogether. At the same time, scFv425:sTRAILmR1-5 treated cells showed increased levels of NFκB activation, as judged by IL-8 production. Therefore, although $cFLIP_L$ has been predominantly described as a constitutive activator of NFκB signaling our data actually highlight a potential opposite correlation, with loss of $cFLIP_L$ occurring concurrently with higher NFκB activity. This finding is in agreement with a previous report

by Wajant et al., who also demonstrated an inhibitory role of $cFLIP_L$. A further intriguing finding regarding the NFκB pathway, is the differential processing of the pro-units p100 into p52 by scFv425:sTRAIL-wt but not by scFv425:sTRAILmR1-5. Although puzzling, our data highlight the very complex relationship between TRAIL-receptor signaling and NFκB activation.

Of note, mutant sTRAILmR1-5 is a derivative of another designed TRAIL-R1 selective sTRAIL mutant, sTRAILmR1-6, that was generated by Kelley et al. (23). Based partly on mutant sTRAILmR1-6, Kelley et al. ascribed a greater role for TRAIL-R2 in TRAIL-apoptotic signaling in solid tumors. Since this mutant was later found to be inactive and we here clearly demonstrate TRAIL-R1 signaling in solid tumor cells, the conclusions of Kelley et al. that TRAIL-R1 is not important in solid tumors may need to be revised.

Although homotrimeric wildtype sTRAIL is widely regarded as non-toxic towards normal human cells, an important issue to consider in the use of mutated versions of sTRAIL, such as sTRAILmR1-5, is the potential presence of toxicity resulting from the incorporated amino acid mutations. A future in depth toxicological evaluation in primary human cells and in appropriate animal models will be required to accurately assess the toxicity profile, and thus the therapeutic potential, of scFv425:sTRAILmR1-5.

In conclusion, the EGFR-specific delivery of mutant sTRAILmR1-5 results in superior apoptotic activity towards EGFR-positive tumor cells. Further preclinical development of scFv425:sTRAILmR1-5 appears warranted to determine the therapeutic potential of this fusion protein for the treatment of EGFR-positive tumors.

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Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP)-targeted delivery of soluble TRAIL potently inhibits melanoma outgrowth in vitro and in vivo.

Marco de Bruyn[1], Anna Rybczynska[2], Yunwei Wei[1], Michael Schwenkert[3], Georg Fey[3], Rudi Dierckx[2], Aren van Waarde[2], Wijnand Helfrich[1] and Edwin Bremer[1]

[1]Department of Surgery, Surgical Research Laboratories, University Medical Center Groningen, University of Groningen, Hanzeplein 1, 9713GZ, Groningen, The Netherlands.

[2]Nuclear Medicine and Molecular Imaging, University of Groningen Medical Center, University of Groningen, Hanzeplein 1, 9713GZ, Groningen, The Netherlands.

[3]Chair of Genetics, University of Erlangen-Nuremberg, Erlangen, Germany

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Abstract

Advanced melanoma is characterized by a pronounced resistance to therapy leading to a limited patient survival of ~6-9 months. Here, we report on a novel bifunctional therapeutic fusion protein, designated anti-MCSP:TRAIL, that is comprised of a melanoma-associated chondroitin sulfate proteoglycan (MCSP)-specific antibody fragment (scFv) fused to soluble human TRAIL. MCSP is a well-established target for melanoma immunotherapy and has recently been shown to provide important tumorigenic signals to melanoma cells. TRAIL is a highly promising tumoricidal cytokine with no or minimal toxicity towards normal cells. Anti-MCSP:TRAIL was designed to 1. selectively accrete at the cell surface of MCSP-positive melanoma cells and inhibit MCSP tumorigenic signaling and 2. activate apoptotic TRAIL-signaling. Treatment of a panel of MCSP-positive melanoma cell lines with anti-MCSP:TRAIL induced TRAIL-mediated apoptotic cell death within 16h. Of note, treatment with anti-MCSP:TRAIL was also characterized by a rapid dephosphorylation of key proteins, such as FAK, implicated in MCSP-mediated malignant behavior. Importantly, anti-MCSP:TRAIL treatment already inhibited anchorage-independent growth by 50% at low picomolar concentrations, whereas >100 fold higher concentrations of non-targeted TRAIL failed to reduce colony formation. Daily i.v. treatment with a low dose of anti-MCSP:TRAIL (0.14mg/kg) resulted in a significant growth retardation of established A375M xenografts. Anti-MCSP:TRAIL activity was further synergized by co-treatment with rimcazole, a σ-ligand currently in clinical trials for the treatment of various cancers. In conclusion, anti-MCSP:TRAIL has promising pre-clinical anti-melanoma activity that appears to result from combined inhibition of tumorigenic MCSP-signaling and concordant activation of TRAIL-apoptotic signaling. Anti-MCSP:TRAIL alone, or in combination with rimcazole, may be of potential value for the treatment of malignant melanoma.

Background

Patients diagnosed with localized melanoma have a 10-year survival rate of up to 95%. In contrast, the life expectancy of patients with metastasized melanoma is limited to only 6-9 months [1]. The poor survival of patients with advanced melanoma is largely attributable to resistance towards current treatment modalities such as chemo- and radiotherapy [2]. Therefore, the development of novel therapeutic approaches that can trigger melanoma-selective cell death appears warranted.

One target molecule of potential relevance for the tumorigenicity of melanoma is the melanoma chondroitin sulfate proteoglycan (MCSP), also known as high molecular weight melanoma associated antigen (HMW-MAA). MCSP is highly expressed on the cell surface of both benign dysplastic nevi and on over 85% of malignant melanomas [3]. Important-

ly, over-expression of MCSP correlates with an unfavorable prognosis [4]. Expression of MCSP on normal tissue is mainly restricted to cells of the melanocyte lineage, but has also been detected in basal cells of the epidermis and within the hair follicle, in pericytes, chondrocytes and smooth muscle cells [3]. Recent studies have revealed that MCSP expression may provide important tumorigenic signals to melanoma cells. MCSP signaling stimulates growth, motility, and tissue invasion by melanoma cells, e.g. by enhancing integrin function [5], activation of Focal Adhesion Kinase (FAK) [6], mitogenic ERK signaling [7] and matrix metalloproteinase 2 [8]. Furthermore, non-metastatic radial growth tumor cells acquired anchorage-independent growth characteristics upon ectopic expression of MCSP [6]. Of note, anti-MCSP antibody treatment can partly inhibit MCSP-tumorigenic signaling in vitro, as evidenced by a pronounced inhibition of FAK [6].

Thus, MCSP appears to be important for melanoma tumorigenicity and appears to be a promising target for both naked monoclonal antibody (mAb) as well as immunotoxin-based strategies [9,10]. Notably, anti-MCSP mAbs proved to have beneficial effects on the clinical course of the disease of melanoma patients [3,11].

In recent years, we have demonstrated that scFv antibody fragment-targeted delivery of the immuno cytokine TRAIL holds particular promise for tumor-selective induction of apoptosis in various cancer types. TRAIL (Tumor Necrosis Factor Related Apoptosis Inducing Ligand) is a highly promising anti-cancer agent with pronounced pro-apoptotic activity towards various malignant cell types, including melanoma. Importantly, TRAIL essentially lacks activity towards normal cells [12]. Based on these characteristics, recombinant soluble TRAIL (sTRAIL) preparations have recently entered clinical trials, with promising preliminary reports on anti-tumor activity and safety [13].

Antibody fragment-mediated targeting of TRAIL can further selectively enhance the anti-tumor activity of TRAIL towards various types of cancer, including carcinomas and Acute Myeloid Leukemia [12] [14-20] (see also chapter 2). Briefly, genetic fusion of TRAIL to an scFv antibody fragment allows for the selective delivery of TRAIL to a pre-selected tumor-associated antigen at the tumor cell surface. The resulting high levels of tumor cell surface bound TRAIL then efficiently activate apoptotic signaling via the agonistic TRAIL-receptors TRAIL-R1 and TRAIL-R2 in a mono- and/or bi/multicellular manner [14-18,20]. Of note, non-targeted sTRAIL has no intrinsic tumor-selective binding activity and is less efficient in cross-linking and activating TRAIL-R2 [21].

Here we preclinically evaluated the anti-melanoma activity of MCSP-targeted delivery of TRAIL, using fusion protein anti-MCSP:TRAIL. Anti-MCSP:TRAIL was designed to selectively bind to MCSP at the cell surface of melanoma cells and simultaneously inhibit tumorigenic signaling by MCSP. Once bound to MCSP-expressing melanoma cells, the anti-MCSP:TRAIL fusion protein can activate apoptotic TRAIL-signaling. Since TRAIL resistance has been reported for melanoma [22] we further evaluated a combinatorial strat-

egy in which anti-MCSP:TRAIL treatment was combined with rimcazole. Rimcazole is a sigma ligand currently in clinical trials for various cancers that has shown potent single-agent anti-tumor activity towards glioma and breast cancer [23,24]. Sigma ligand-based therapy may also be of value for the treatment of melanoma since σ-receptors 1 and 2 are strongly overexpressed in this tumor [25,26].
MCSP targeting with anti-MCSP:TRAIL indeed appeared to inhibit MCSP-signaling and activated TRAIL-apoptotic signaling in melanoma cells *in vitro* and *in vivo*. Furthermore, TRAIL-apoptotic signalling in melanoma cells can be further increased by combinatorial treatment with rimcazole. The dual anti-melanoma activity of anti-MCSP:TRAIL alone, or in combination with rimcazole, may be of interest for treatment of melanoma.

Materials and Methods

Reagents

MAb 9.2.27 is a murine IgG2a with high binding affinity for human MCSP [10]. TRAIL-neutralizing mAb 2E5 was purchased from Alexis (10P's, Breda, The Netherlands). Caspase inhibitors zVAD-fmk and zLEHD-fmk were from Calbiochem (VWR International B.V., Amsterdam, The Netherlands) and dissolved at 10 mM in DMSO. Rimcazole (BW 239U) and (+)pentazocine were from Sigma Aldrich (Sigma-Aldrich Chemie B.V. Zwijndrecht, Netherlands). Stock solutions of rimcazole (3.9 mM in ethanol) and pentazocine (10 mM in 0.1N hydrochloric acid) were freshly prepared for each experiment.

Cell lines

MCSP-positive/EpCAM-negative melanoma cell lines A375M, A2058 and SK-MEL-28 were obtained from and characterized (STR profiling, karyotyping, isoenzyme analysis) by the American Tissue Culture Collection (ATCC). The MCSP-negative melanoma cell line M14 and transfectant cell line M14.MCSP were provided by Prof. Dr. G.H. Fey. Expression of MCSP was determined by flow cytometry using anti-MCSP mAb 9.2.27 and a FITC-conjugated goat-anti-mouse mAb. A375M.FADD-DED cells were generated by transfection of parental A375M cells using Fugene (Roche BV, Woerden, The Netherlands). All cell lines were cultured at 37°C, in a humidified 5% CO2 atmosphere. A375M, A2058, SK-MEL-28 and M14 cells were cultured in RPMI 1640 medium (Cambrex Bio Science, Verviers, France) supplemented with 10% fetal calf serum. M14.MCSP cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 400 μg/ml Geneticin. A375M.FADD-DED cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 500 μg/ml Geneticin.

Primary human hepatocytes (PHH) and melanocytes

Cryopreserved human hepatocytes and melanocytes (Tebu-bio bv, Heerhugowaard, The

Netherlands) were isolated according to standard protocol using hepatocyte isolation kit and melanocyte isolation kit, respectively (tebu-bio bv, Heerhugowaard, The Netherlands). For experiments, hepatocytes and melanocytes were seeded in a 48 well plate at a density of $0.5x10^6$ cells/ml.

Construction of fusion protein anti-MCSP:TRAIL

A scFv antibody fragment in the VH-VL format was derived from mAb 9.2.27 using standard antibody-phage display technology [10]. Fusion protein anti-MCSP:TRAIL was constructed by cloning the cDNA of antibody fragment MCSP in frame with soluble TRAIL into in-house constructed vector pEE14 using unique SfiI and NotI restriction enzyme sites, yielding plasmid pEE14-anti-MCSP:TRAIL. Fusion protein anti-MCSP:TRAIL was produced in CHO-K1 cells using previously described methods [14,16]. Culture medium containing anti-MCSP:TRAIL was cleared by centrifugation (10.000 g, 10 min), filter sterilized, and stored at -80°C until further use. Fusion protein anti-MCSP:TRAIL was purified via the N-terminal Hemagglutinin (HA)-tag using anti-HA affinity chromatography. Fusion protein scFvC54:sTRAIL [14], for reasons of clarity hereafter renamed into anti-EpCAM:TRAIL, is a fusion protein that is essentially identical to anti-MSCP:TRAIL except that it contains an anti-EpCAM scFv instead of an anti-MSCP scFv.

Assessment of apoptosis

MCSP-restricted induction of apoptosis by anti-MCSP:TRAIL was assessed on a panel of melanoma cell lines. Briefly, melanoma cells were pre-cultured in a 48-well plate at a concentration of $3.0x10^4$ cells/well. Subsequently, cells were treated as indicated and apoptosis was assessed by: Loss of mitochondrial membrane potential ($\Delta\psi$); $\Delta\psi$ was analyzed by DiOC6 staining (Eugene, The Netherlands) as previously described [16]. After 16 h treatment, cells were harvested and incubated for 20 min with DiOC6 (0,1 μM) at 37°C, harvested (1000 g, 5 min.), resuspended in PBS, and assessed for staining by flow cytometry. Caspase-8, caspase-9 and caspase-3/-7 activity; caspase activity was assessed using the Caspase-Glo 8, 9 and 3/7 Assay according to manufacturer's instructions (Promega Benelux BV, Leiden, The Netherlands). The assay is based on the cleavage of non-luminescent substrates by activated caspases into a luminescent product. Luminescence was quantified using a Victor3 multi-label plate reader (Perkin Elmer, Groningen, The Netherlands).

Soft agar colony forming assay

Soft agar colony forming assays were performed in 24-well plates pre-coated with 0.5 ml solidified 0.4% agarose in RPMI 1640 medium. Cells were resuspended at a density of $10x10^3$ cells/well in 0,6% agarose in RPMI 1640 medium supplemented with 20% fetal calf serum and layered on the solidified 0.4% agarose in a 24-well plate. Tumor cell contain-

ing agarose was allowed to solidify for 10 minutes at 4°C before addition of 1 ml of RPMI 1640 medium supplemented with 20% fetal calf serum. Cells were treated as indicated and subsequently cultured at 37°C, in a humidified 5% CO2 atmosphere. Colony formation was assessed 21 days after start of treatment. Assays were performed in quadruplicate. Number of colonies was quantified and percentage of colony growth was calculated with the formula: Percentage of colony growth = (number of colonies in experimental condition) / (number of colonies in medium control) x 100%. For A375M cells, the number of colonies in untreated samples ranged from 30±5 to 262±35 between individual experiments.

Proteome Profiler array

The effect of MCSP targeting on protein tyrosine kinase activity was assessed by the Human Phospho-Kinase Array Kit (R&D Systems) according to the manufacturer's recommendations. Briefly, per condition 1 x 10^7 cells were treated for 0, 30, 60, or 240 min. at 37°C as indicated. Lysate (500 µg/condition) was added to the NC-membranes containing spotted Phospho-Kinase (PK) antibodies. Subsequently, secondary Biotin-conjugated anti-Kinase antibodies and Horseradish Peroxidase-conjugated Streptavidin were added to detect the presence of phosphorylated kinases. Between incubation steps, membranes were rigorously washed. Blots were developed by standard chemoluminescence (Roche). Luminescent signals were quantified using the IVIS Spectrum bioluminescent imager (Caliper Biosciences) as photons/sec/sr/cm^2. After background luminescence subtraction, the relative kinase activity in treated conditions was calculated by the formula: (PK activity experimental condition / PK activity medium control)*100%.

A375M xenograft mouse model

Experiments involving animals were performed in accordance with the experimental protocol approved by the Committee for Research and Animal Ethics of the UMCG. Six week old healthy male athymic mice (n=8) were purchased from Harlan (Harlan Netherlands B.V., Horst, The Netherlands). Mice were housed in IVC cages and fed ad libitum. Subsequently, mice were subcutaneously inoculated with 2 x 10^6 A375M cells suspended in 100 µl matrigel. Tumor growth was monitored daily by electronic caliper measurements. After reaching tumor size of ~50 ± 6 mm^3, mice were randomly assigned into two groups with a sample size of 4. Mice received daily i.v. saline injections or anti-MCSP:TRAIL injections (0.14 mg/kg). After two weeks of treatment, mice were sacrificed by cervical dislocation. Tumor size was calculated by the formula: V = 0,5234 x H x L x W [mm^3]. Tumor size was expressed as the percentage of maximum tumor size in Sham-treated mice.

Liver histopathology

Formalin-fixed liver samples were embedded in paraffin, sectioned into 5-µm thickness and

stained with hematoxylin-eosin and microscopically inspected for hepatic tissue damage and inflammation.

Combination treatment with anti-MCSP:TRAIL and σ-ligands
Cells were plated at 3.0 x 10^4 cells / well in a 48-well plate and allowed to adhere overnight. Cells were concurrently treated for 24h with anti-MCSP:TRAIL with or without rimcazole (15 μM), and (+)pentazocine (200 nM). Synergy was assessed by calculating the cooperativity index (CI) in which the sum of apoptosis induced by single-agent treatment is divided by apoptosis induced by combination treatment. When CI was less than 1, treatment was considered synergistic; when CI equaled 1, treatment was considered additive; when CI was greater than 1, treatment was considered antagonistic.

Statistical analysis
Data reported are mean values ± standard error of the mean of at least three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer post test or by two-sided unpaired Student's t-test. $p < 0.05$ was defined as a statistically significant difference.

Results and Discussion
MCSP-restricted induction of apoptosis by anti-MCSP:TRAIL
We and others have previously demonstrated that selective delivery of the cytokine TRAIL by genetic fusion to an appropriate tumor-selective scFv antibody fragment significantly enhances the anti-tumor activity of TRAIL towards the corresponding type of cancer [14-20]. Here, we adapted this targeted approach to malignant melanoma by exploiting an ant-MCSP scFv antibody fragment that was derived from the well-established monoclonal antibody (mAb) 9.2.27. The resultant fusion protein, anti-MCSP:TRAIL, was equipped to selectively accrete at the cell surface of MCSP-positive cells only and subsequently trigger TRAIL-mediated apoptosis.
Indeed, treatment of MCSP-transfected M14 melanoma cells (M14.MCSP) with anti-MCSP:TRAIL resulted in dose-dependent activation of apoptosis within 16h, whereas parental MCSP-negative M14 cells were resistant to treatment with anti-MCSP:TRAIL (Fig.1A). Similarly, treatment of a series of MCSP-positive cell lines (A375M, A2058 and SK-MEL-28) with anti-MCSP:TRAIL resulted in a marked induction of apoptosis (Fig. 1B). In a control experiment we treated the same series of melanoma cells with fusion protein anti-EpCAM:TRAIL, a fusion protein that is essentially identical to anti-MCSP:TRAIL except that it contains an anti-EpCAM scFv instead of an anti-MCSP scFv antibody fragment [14]. EpCAM is a well established carcinoma-associated cell surface target antigen that is not expressed on melanoma cells. Indeed, no signs of apoptosis were observed when A375M,

A2058 or SK-MEL-28 cells were treated with anti-EpCAM:TRAIL (Fig. 1C and D).
Treatment of MCSP-positive A375M, A2058 or SK-MEL-28 cells with the parental anti-MCSP antibody mAb 9.2.27 failed to induce any signs of apoptosis (Fig.1D). Importantly, also co-treatment with MAb 9.2.27 and anti-EpCAM:TRAIL did not induce any significant signs of apoptosis (Fig. 1D). Thus, treatment of MCSP-positive melanoma cells with fusion protein anti-MCSP:TRAIL efficiently activates apoptosis that is MCSP-restricted and that is superior to combined treatment with TRAIL and the parental anti-MCSP mAb 9.2.27.
Induction of apoptosis by anti-MCSP:TRAIL was abrogated when treatment was performed in the presence of the parental mAb 9.2.27 (Fig. 2A). Thus, anti-MCSP:TRAIL activity is strictly dependent on binding to cell surface-expressed MCSP on malignant cells. Furthermore, addition of the TRAIL-neutralizing mAb 2E5 fully abrogated the apoptotic effect of anti-MCSP:TRAIL (Fig.2A), indicating that induction of apoptosis is dependent on interaction of TRAIL with its agonistic TRAIL-receptors. In addition, induction of apoptosis was inhibited when treatment is performed in the presence of pan-caspase-inhibitor zVAD-fmk (Fig. 2A), further suggesting involvement of TRAIL-mediated apoptotic signaling. Indeed, treatment of melanoma cells with anti-MCSP:TRAIL was characterized by time-dependent activation of initiator caspase-8 (Fig. 2B) as well as effector caspases 3 and 7 (Fig. 2C).
To further confirm that TRAIL-signaling is involved, the mutant cell line A375M.FADD-DED that ectopically overexpresses a dominant negative mutant of the adaptor protein FADD, was produced. FADD is a pivotal adaptor protein in TRAIL-receptor signaling. The mutant FADD protein that was ectopically expressed in A375M.FADD-DED cells lacks the so-called DED domain, which results in a general and strong resistance against TRAIL-mediated apoptosis. Indeed, TRAIL-mediated apoptotic signaling was fully halted in A375M.FADD-DED cells, as is evidenced by the fact that these cells were fully resistant to ubiquitously active recombinant human TRAIL (rhTRAIL) (Fig. 2D). Likewise, A375M.FADD-DED cells were also fully resistant to treatment with anti-MCSP:TRAIL (Fig. 2D). Taken together, these data clearly demonstrate that the pro-apoptotic activity of anti-MCSP:TRAIL is TRAIL-mediated.
Previously, we and others have shown that upon binding to the respective target antigen, scFv:TRAIL fusion proteins can efficiently activate TRAIL-R2, whereas untargeted sTRAIL preparations have a markedly reduced capacity to activate TRAIL-R2 signaling. Indeed A375M cells, that solely express TRAIL-R2 at the cell surface (Fig. 3A) are sensitive to treatment with anti-MCSP:TRAIL (Fig. 3B), indicating that anti-MCSP:TRAIL can potently activate TRAIL-R2 signaling in MCSP-positive melanoma cells. This feature may be of special relevance for melanoma since TRAIL-R2 is the most prevalent agonistic TRAIL-receptor expressed on melanoma cells [27].

Potent inhibition of anchorage-independent growth by anti-MCSP:TRAIL

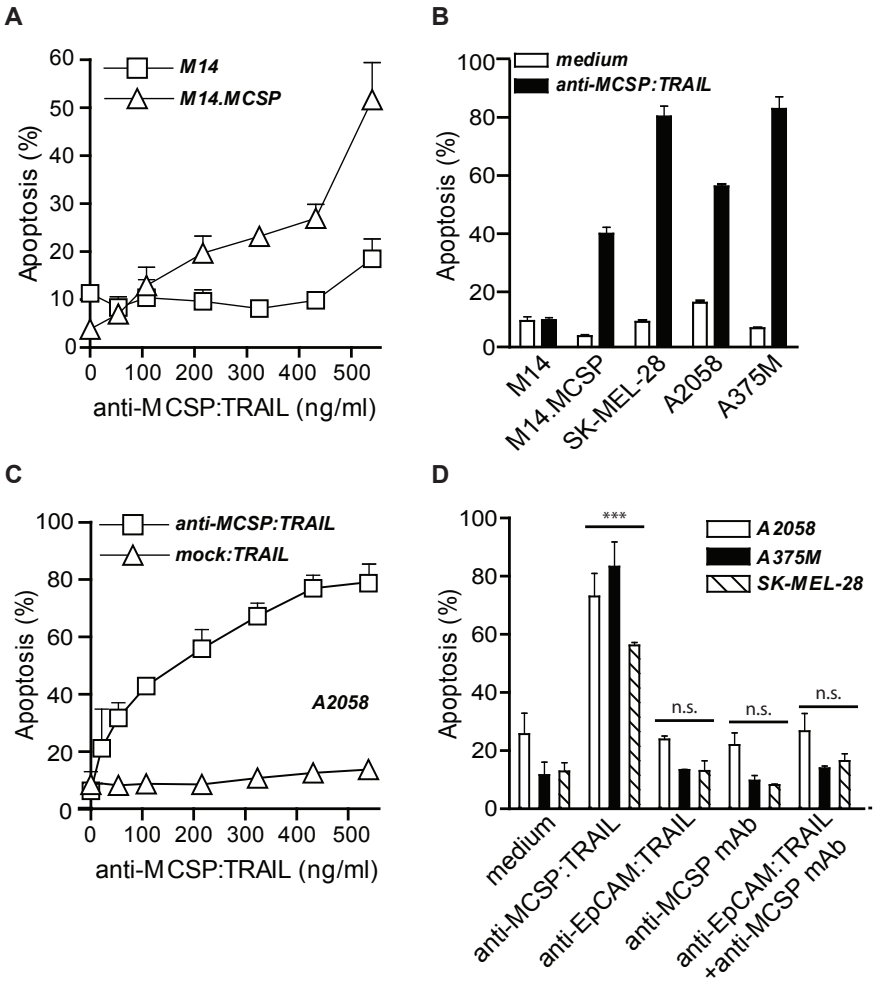

Figure 1. MCSP-restricted induction of apoptosis by anti-MCSP:TRAIL A M14 and M14.MCSP cells were treated with increasing concentrations of anti-MCSP:TRAIL for 16h and apoptosis was assessed. **B** MCSP-negative cell line M14 and MCSP-positive cell lines M14.MCSP, SK-MEL-28, A2058 and A375M were treated with 500 ng/ml anti-MCSP:TRAIL for 16h and apoptosis was assessed. **C** A2058 cells were treated with increasing concentrations of anti-MCSP:TRAIL or anti-EpCAM:TRAIL for 16h and apoptosis was assessed **D** A375M, A2058 and SK-MEL-28 cells were treated with equimolar concentrations of anti-MCSP:TRAIL, anti-EpCAM:TRAIL, anti-MCSP mAb or anti-EpCAM:TRAIL+anti-MCSP mAb for 16h and apoptosis was assessed. In all experiments, apoptosis was assessed by $\Delta\psi$

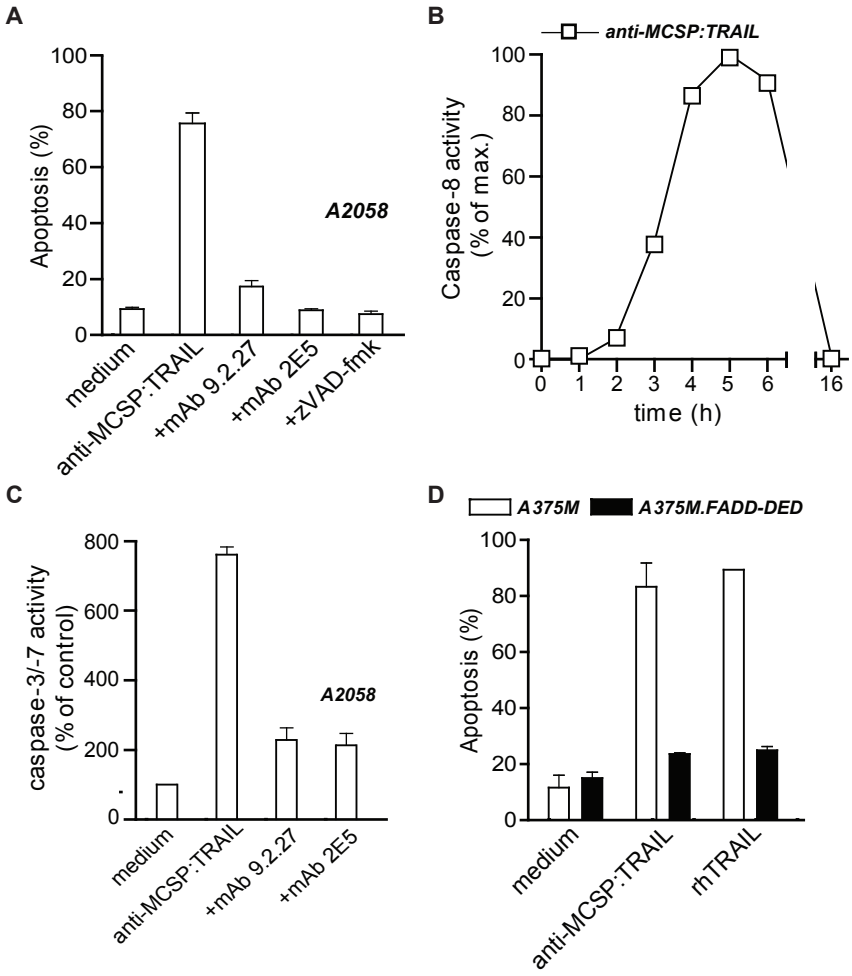

Figure 2. Caspase-dependent induction of apoptosis by anti-MCSP:TRAIL A A2058 cells were treated with 500 ng/mL anti-MCSP:TRAIL in the absence or presence of parental MCSP-blocking mAb 9.2.27, TRAIL-neutralizing mAb 2E5 or pan-caspase inhibitor zVAD-fmk for 16h and apoptosis was assessed. **B** A375M cells were treated with 500 ng/mL anti-MCSP:TRAIL for the time-points indicated and caspase-8 activation was assessed. **C** A2058 cells were treated with 500 ng/mL anti-MCSP:TRAIL in the absence or presence of parental MCSP-blocking mAb 9.2.27 or TRAIL-neutralizing mAb 2E5 and caspase-3/-7 activation was assessed. **D** A375M and A375M.FADD-DED cells were treated with anti-MCSP:TRAIL or rhTRAIL for 16h and apoptosis was assessed. In all experiments, apoptosis was assessed by $\Delta\psi$.

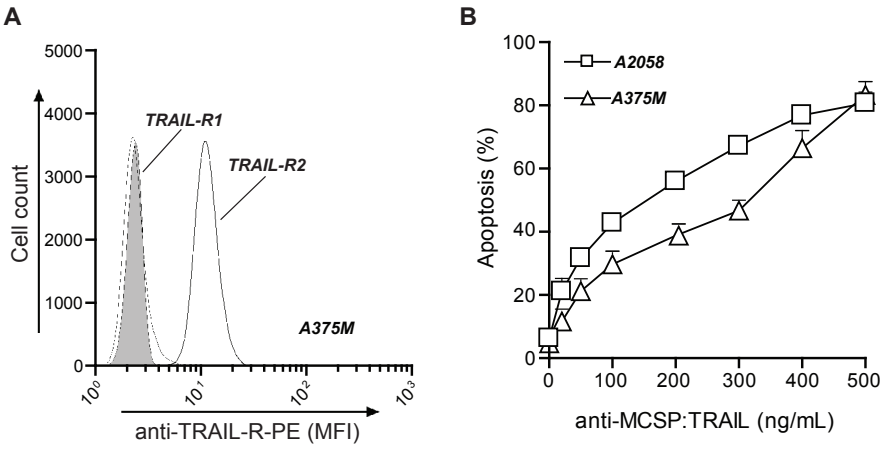

Figure 3. Anti-MCSP:TRAIL induces apoptosis in TRAIL-R1 negative cell lines. A A375M cells were incubated with anti-TRAIL-R1 (thin line) or anti-TRAIL-R2 (thick line) mAb and expression of TRAIL-R1 and TRAIL-R2 was analyzed by flow cytometry. Shaded area indicates the fluorescence signal when cells were incubated with fluorescent-labeled secondary antibody alone. **B** A2058 and A375M cells were treated with increasing concentrations of anti-MCSP:TRAIL for 16h and apoptosis was assessed by $\Delta\psi$.

The MCSP antibody fragment used in this study was derived from mAb 9.2.27. In earlier studies, mAb 9.2.27 partly inhibited MCSP-tumorigenic signaling *in vitro*, as evidenced by inhibition of FAK [9]. Since ectopic expression of MCSP was also recently shown to induce anchorage-independent growth capacity, anti-MCSP:TRAIL was evaluated for its effect on anchorage-independent growth of melanoma cells. In a soft agar assay, treatment of the MCSP-positive cell lines A375M and A2058 cells with anti-MCSP:TRAIL abrogated colony outgrowth in a dose-dependent manner (Fig.4A). Of note, anti-MCSP:TRAIL reduced colony formation by 50% already at very low concentrations (0.1 ng/ml and 1ng/ml for A375M and A2058 cells, respectively). Induction of apoptosis by anti-MCSP:TRAIL at these concentrations in adherent growth conditions was marginal (see Fig. 3B for dose-response curves of apoptosis induction). In contrast, treatment with similar concentrations of anti-EpCAM:TRAIL or with anti-MCSP mAb 9.2.27 only marginally reduced colony formation (Fig. 4B). Importantly, also combinatorial treatment with equimolar concentrations of anti-EpCAM:TRAIL and mAb 9.2.27 failed to significantly inhibit colony outgrowth of A375M cells at the highest concentrations tested (Fig.4B). Furthermore, rhTRAIL failed to significantly reduce colony outgrowth (Fig. 4C). Thus, anti-MCSP:TRAIL uniquely and efficiently prevents colony outgrowth.

Treatment of A375M cells with anti-MCSP:TRAIL in the presence of the TRAIL-neutralizing mAb 2E5 largely blocked the effect of anti-MCSP:TRAIL on colony outgrowth (Fig.4D).

Similarly, treatment of cells with anti-MCSP:TRAIL in the presence of the parental anti-MCSP mAb 9.2.27 inhibited anti-MCSP:TRAIL activity, although the number of colonies still remained significantly lower than medium control (Fig.4D). Of note, co-incubation with pan-caspase inhibitor zVAD-fmk abrogated the inhibitory effect of anti-MCSP:TRAIL on colony outgrowth (Fig.4D), suggesting that apoptotic signaling is required for the inhibitory effect of anti-MCSP:TRAIL.

To further evaluate whether TRAIL-induced caspase-signaling was required for the inhibitory effect of anti-MCSP:TRAIL on colony outgrowth, TRAIL-resistant A375M.FADD-DED cells were treated with anti-MCSP:TRAIL. Importantly, colony formation of A375M.FADD-DED cells was inhibited by anti-MCSP:TRAIL treatment although ~50-100 fold higher concentrations were required to obtain the same magnitude of inhibition as observed for parental A375M cells (Fig. 5A). Of note, treatment of A375M.FADD-DED cells with anti-EpCAM:TRAIL did not inhibit colony formation (Fig. 5B), nor did combination treatment of anti-EpCAM:TRAIL and mAb 9.2.27 (Fig. 5B). In contrast, mAb 9.2.27 alone did significantly inhibit colony outgrowth in this cell line, although to a lesser extent than anti-MCSP:TRAIL (Fig. 5B). Together, these experimental data suggest that the simultaneous interaction of anti-MCSP:TRAIL with MCSP and TRAIL-receptors uniquely blocks anchorage-independent growth of melanoma cells at low concentrations.

Anti-MCSP:TRAIL dephosphorylates proteins involved in cell proliferation and apoptosis resistance

The efficacy of anti-MCSP:TRAIL on inhibition of colony formation by melanoma cells in 3D-culture suggests that antibody fragment-dependent sensitization of cells to concurrent TRAIL-signaling may contribute to its therapeutic effect. Previously, we generated proof of concept data for such dual therapeutic signaling with scFv:TRAIL fusion proteins, using an scFv:TRAIL fusion protein that targeted the Epidermal Growth Factor Receptor [16,19] (see also chapter 3 and 4). Here, the EGFR-inhibitory antibody fragment blocked mitogenic EGFR-signaling and synergized TRAIL apoptotic signaling [16,19]. To assess whether the MCSP-specific antibody fragment might contribute to the anti-tumor activity of anti-MCSP:TRAIL, a focused set of 48 cellular phosphoproteins was examined for the level of phosphorylation after treatment with anti-MCSP:TRAIL (Fig.6A, B), anti-MCSP mAb 9.2.27 (Fig. 6C) and rhTRAIL (Fig. 6D). Interestingly, although A375M cells were treated with anti-MCSP:TRAIL in adherent growth conditions in view of experimental limitations with 3D-cultures, significant dephosphorylation of various proteins involved in cell survival and proliferation was detected after 1h of treatment (Fig.6A, B). This dephosphorylation induced by anti-MCSP:TRAIL was detectable within 30 minutes and increased for up to 4h after start of treatment (Fig. 6E). Treatment with mAb 9.2.27 and rhTRAIL also triggered dephosphorylation of proteins at 1h of treatment, but to a lesser extent and in a smaller

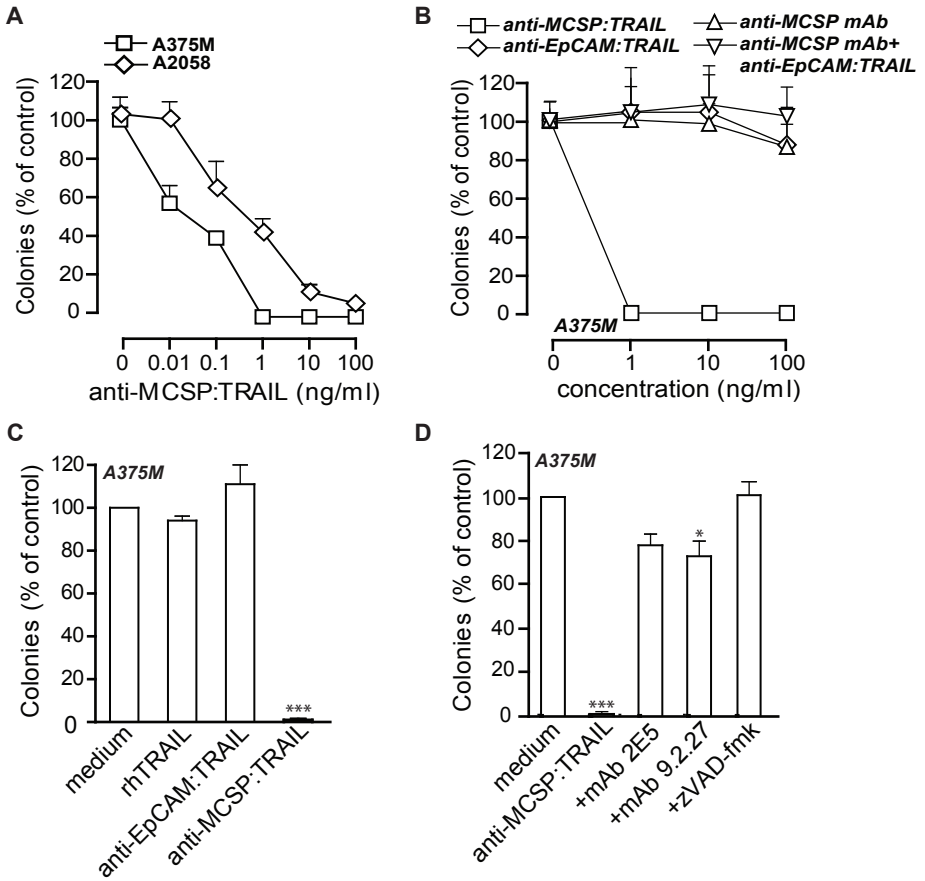

Figure 4. Inhibition of anchorage-independent growth by anti-MCSP:TRAIL. A A375M and A2058 cells were treated with increasing concentrations of anti-MCSP:TRAIL for 21 days and colony formation was assessed. **B** A375M cells were treated with increasing concentrations of anti-MCSP:TRAIL, anti-EpCAM:TRAIL, anti-MCSP mAb or anti-EpCAM:TRAIL+anti-MCSP mAb for 21 days and colony formation was assessed. **C** A375M cells were treated with rhTRAIL, anti-EpCAM:TRAIL or anti-MCSP:TRAIL for 21 days and colony formation was assessed. **D** A375M cells were treated with 100 ng/ml anti-MCSP:TRAIL in the absence or presence of TRAIL-neutralizing mAb 2E5, parental anti-MCSP mAb 9.2.27 or pan-caspase inhibitor zVAD-fmk for 21 days and colony formation was assessed.

panel (Fig. 6C and D, respectively).

First and foremost, the previously reported down-stream target of MCSP, FAK, was dephosphorylated by a factor 2 compared to medium control. The parental anti-MCSP mAb 9.2.27 has been shown to prevent association of MCSP with integrins, thereby preventing activation of FAK [6]. Thus, although not determined here for anti-MCSP:TRAIL, a similar

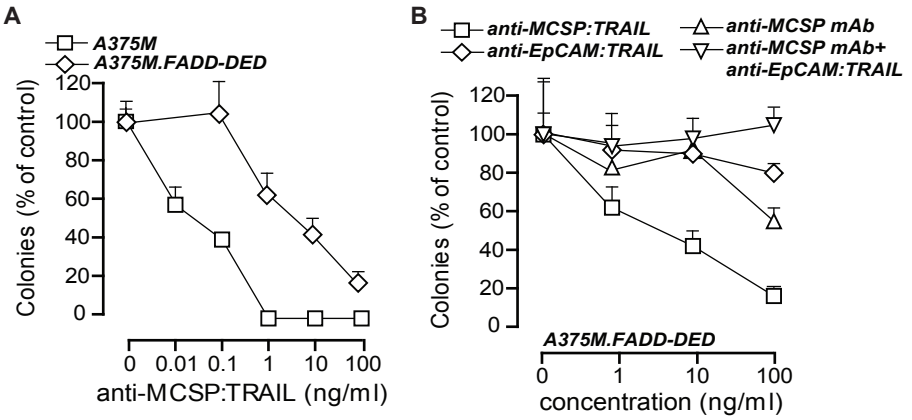

Figure 5. Inhibition of anchorage-independent growth by anti-MCSP:TRAIL partly relies on TRAIL-independent signaling. A A375M and A375M.FADD-DED cells were treated with increasing concentrations of anti-MCSP:TRAIL for 21 days and colony formation was assessed. **B** A375M.FADD-DED cells were treated with increasing concentrations of anti-MCSP:TRAIL, anti-EpCAM:TRAIL, anti-MCSP mAb or anti-EpCAM:TRAIL+anti-MCSP mAb for 21 days and colony formation was assessed.

inhibition of MCSP / integrin interaction by the mAb 9.2.27-derived antibody fragment may be responsible for the inhibitory effect of anti-MCSP:TRAIL on FAK. Intriguingly, cross-linking of MCSP by bead-coated mAb 9.2.27 has also been used to achieve activation of MCSP-signaling [6]. The different outcome of treatment with soluble vs. coated mAb suggests that the extent of cross-linking of MCSP by the antibody determines the activation or inhibition, respectively, of downstream signaling. It will be interesting to evaluate in future studies whether the three MCSP reading heads in trimeric anti-MCSP:TRAIL are perhaps better suited for inhibition of MCSP-signaling.

The activity of ERK1/2, another established downstream effector of MCSP signaling [6], was not affected after 1h of treatment with anti-MCSP:TRAIL. One possible reason for this finding is a perhaps delayed effect of anti-MCSP:TRAIL on ERK1/2 signaling at later and not examined time-points. In addition, it has been shown that MCSP enhances FAK and Erk1/2 signaling by distinct mechanisms [6]. It is therefore also conceivable that FAK is dephosphorylated while Erk1/2 is not a target of inhibition by anti-MCSP:TRAIL.

In addition to the established MCSP-target FAK, a panel of other proteins was dephosphorylated upon anti-MCSP:TRAIL treatment, including the kinase Fyn, and the Src kinases Src, Hck, Lyn and Yes. The relative impact of these respective proteins on MCSP tumorigenic signaling is currently being evaluated in extended ongoing studies using e.g. constitutively active and/or dominant negative mutants as well as small inhibitory RNA-mediated silencing of the individual components. Importantly, the above-mentioned experi-

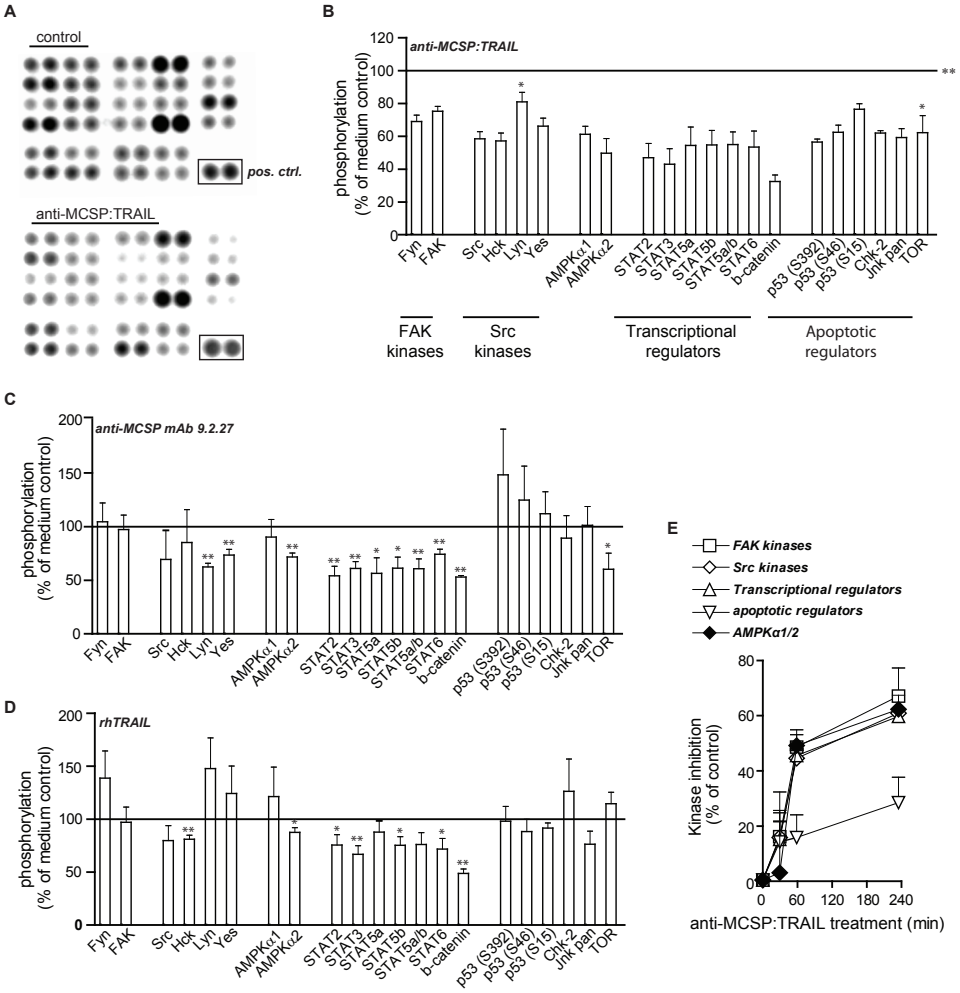

Figure 6. Anti-MCSP:TRAIL triggers dephosphorylation of cellular proteins. A375M cells were treated with anti-MCSP:TRAIL, anti-MCSP mAb 9.2.27, rhTRAIL or left untreated for 1h. Whole cell lysates were analyzed for phosphorylation of 48 cellular kinases. **A** Phosphorylation of 28 kinases in untreated cells (left panel) or anti-MCSP:TRAIL-treated cells (right panel) are depicted. Images are representative of three independent experiments. **B**, **C**, **D** Changes in the phosphorylation of 21 kinases after treatment with **B** anti-MCSP:TRAIL, **C** anti-MCSP mAb 9.2.27 or **D** rhTRAIL are expressed as a percentage of the phosphorylation measured in untreated cells. **E** A375M cells were treated with anti-MCSP:TRAIL for 0, 30, 60 or 240 min. Whole cell lysates were analyzed for phosphorylation of 48 cellular kinases. Changes in phosphorylation of the different kinase groups as indicated in panel B are depicted. *, p<0.05; **, p<0.001; ***, p<0.0001.

ments need to be performed not only in 2D, but more importantly also in 3D-cultures, in order to reliable determine the relative importance of the here identified proteins for MCSP-dependent anchorage-independent growth of melanoma cells.

Perhaps counter intuitively, the proto-oncogene Beta-Catenin (ß-Catenin) was also dephosphorylated by treatment with anti-MCSP:TRAIL, as well as by treatment with mAb 9.2.27 and rhTRAIL (Fig. 6B, C and D, respectively). Dephosphorylation of ß-Catenin does not inactivate ß-Catenin but actually prevents proteasomal degradation and increases cellular levels of ß-Catenin. In turn, this leads to activation of pro-oncogenic gene transcription [28]. However, the exact role of ß-Catenin-induced gene transcription in melanoma is still a matter of debate. Based on mouse models with activating mutations in ß-Catenin, an oncogenic role of ß-Catenin in melanoma was proposed [29]. On the other hand, others have shown that ß-Catenin induces a transcriptional profile in melanoma cells that is reminiscent of normal melanocyte differentiation [30]. This transcriptional profile is moreover associated with increased patient survival and is lost upon malignant progression [30].

Taken together, anti-MCSP:TRAIL dephosphorylates a panel of established MCSP targets as well as newly identified proteins. Thus, the kinase array data support a dual anti-melanoma activity by anti-MCSP:TRAIL that partly relies on inhibition of tumorigenic signaling by the anti-MCSP antibody fragment.

Anti-tumor activity of low dose anti-MCSP:TRAIL towards A375M xenografts

To further characterize the anti-melanoma activity of anti-MCSP:TRAIL, A375M cells were xenografted subcutaneously in nude mice and allowed to form small tumors (~50 mm3). Subsequently, mice were treated daily by intra-peritoneal injection with a low dose of anti-MCSP:TRAIL (~0.14 mg/kg) or with saline. Of note, anti-MCSP mAb 9.2.27 and therefore anti-MCSP:TRAIL do not cross-react with mouse MCSP [3]. Compared to sham-treated mice, tumor size of the anti-MCSP:TRAIL-treated mice was strongly retarded in time (Fig.7A), with a 50% reduction in tumor size at the end of the experiment (Fig.7B). Of note, earlier animal studies with A375M cells and non-targeted rhTRAIL were performed at >300 times higher concentrations (50 mg/kg) compared to the here employed treatment regimen with anti-MCSP:TRAIL [31]. Thus, in analogy to the low dose required for therapeutic activity in colony formation assays, anti-MCSP:TRAIL already has potent *in vivo* anti-melanoma activity at a very low dose.

Anti-MCSP:TRAIL lacks apoptotic activity towards normal cells

Earlier studies have shown that tumor targeted delivery of TRAIL augments the tumor-specific activity of TRAIL, while not affecting the absence of toxicity on normal human cell types. Indeed, although anti-MCSP:TRAIL strongly bound to MCSP on normal human melanocytes (Fig. 8A), no apoptosis was induced in melanocytes even when treatment

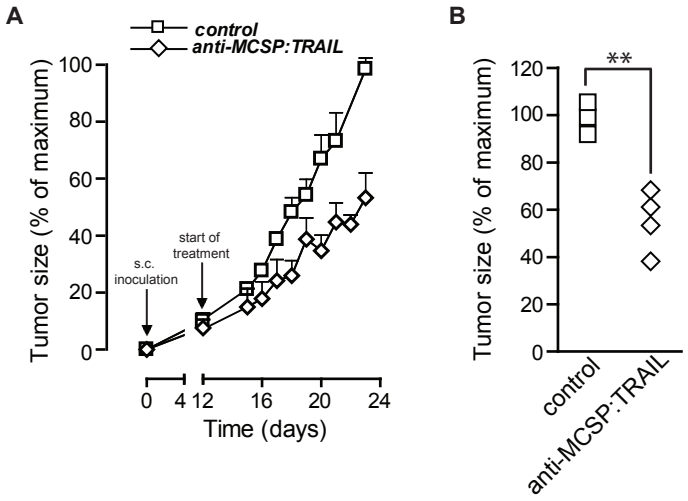

Figure 7. Anti-tumor activity of anti-MCSP:TRAIL towards A375M xenografts. A Mice were inoculated with A375M tumor cells at day 0 and developed xenografts of ~50mm3 after 12 days. Starting at day 12, mice were injected daily with saline (n = 4) or anti-MCSP:TRAIL (0,14 mg/kg, n = 4) and tumor size was measured daily using electronic caliper measurements. **B** Tumor size at day 23.

was performed with 4µg/ml anti-MCSP:TRAIL (Fig. 8B). Also extended treatment (up to 8 days) of melanocytes with 4µg/ml anti-MCSP:TRAIL did not yield significant increases in apoptosis compared to medium control (Fig. 8C). Similarly, normal human MCSP-negative hepatocytes were fully resistant to treatment with anti-MCSP:TRAIL (Fig. 8B). Hepatocytes have previously been shown to be one of the most vulnerable normal cell types to possible TRAIL-related toxicity [32]. Importantly, also in nude mice carrying A375M xenografts treatment with anti-MCSP:TRAIL had no deleterious effect on liver morphology, with morphology of liver sections comparable to the morphology of liver sections of Sham-treated mice (Fig. 8D and E). The here obtained data regarding the absence of activity of anti-MCSP:TRAIL towards normal cell types are in line with the large body of evidence in literature on the preclinical safety profile of sTRAIL. Indeed, ongoing clinical trials with rhTRAIL confirm the relative safety of TRAIL treatment with no serious adverse effects and no dose-limiting toxicity reported to date.

Synergistic induction of apoptosis by anti-MCSP:TRAIL and σ-ligands

Since resistance of melanoma cells towards sTRAIL has previously been reported [22] a combinatorial strategy was evaluated of anti-MCSP:TRAIL with rimcazole, a σ-ligand in clinical trials for various cancers. Sigma receptors are expressed in the central nervous, immune, endocrine, and reproductive systems, but also in peripheral organs like kidney;

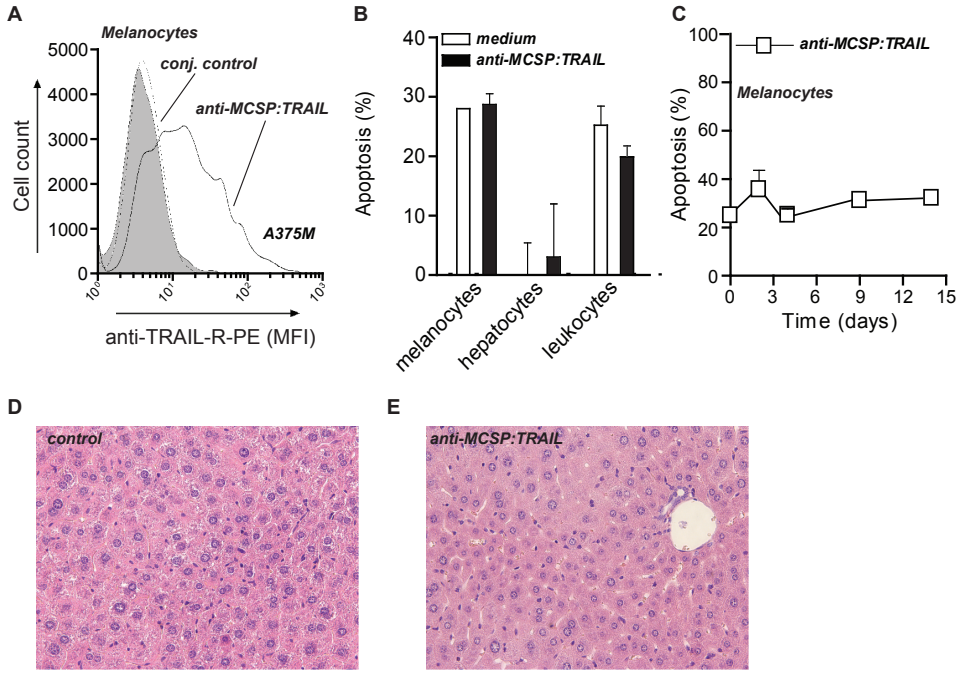

Figure 8. Anti-MCSP:TRAIL lacks apoptotic activity towards normal cells A MCSP-restricted binding of anti-MCSP:TRAIL to melanocytes was assessed by flow cytometry using a PE-conjugated anti-TRAIL mAb. Specific binding was demonstrated by pre-incubating melanocytes with mAb 9.2.27 followed by incubation with anti-MCSP:TRAIL. **B** MCSP-positive melanocytes and MCSP-negative leukocytes and hepatocytes were treated with 500 ng/ml anti-MCSP:TRAIL for 16h and apoptosis was assessed by $\Delta\psi$. **C** Melanocytes were treated with 4 μg/mL anti-MCSP:TRAIL for up to 14 days and apoptosis was assessed by $\Delta\psi$ at time points indicated. **D** Liver pathology of mice carrying A375M xenografts was examined for Sham-treated mice or **E** anti-MCSP:TRAIL-treated mice.

liver; and gastrointestinal tract. Although the precise function of these receptors remains unknown, both σ-R1 and σ-R2 are strongly overexpressed in rapidly proliferating cells such as melanoma and may be exploited as possible targets for melanoma therapy [26]. Treatment of A375M cells with the sigma ligand rimcazole (σ-R1/σ-R2 antagonist) synergistically enhanced induction of apoptosis by anti-MCSP:TRAIL (Fig. 9A and B). Of note, treatment in the presence of the σ-R1 agonist (+)pentazocine did not abrogate synergy, suggesting that rimcazole synergizes with anti-MCSP:TRAIL activity via σ-R2 (Fig. 9C). Rimcazole did not upregulate TRAIL-R1 or TRAIL-R2 expression (data not shown), but augmented the activation of initiator caspase-8 and initiator caspase-9 upon anti-MCSP:TRAIL treatment (Fig. 10A, B, respectively). Moreover, inhibition of either initiator caspase abrogated cytotoxic activity (Fig. 10C). Thus, rimcazole appears to synergize the activity of anti-MCSP:TRAIL

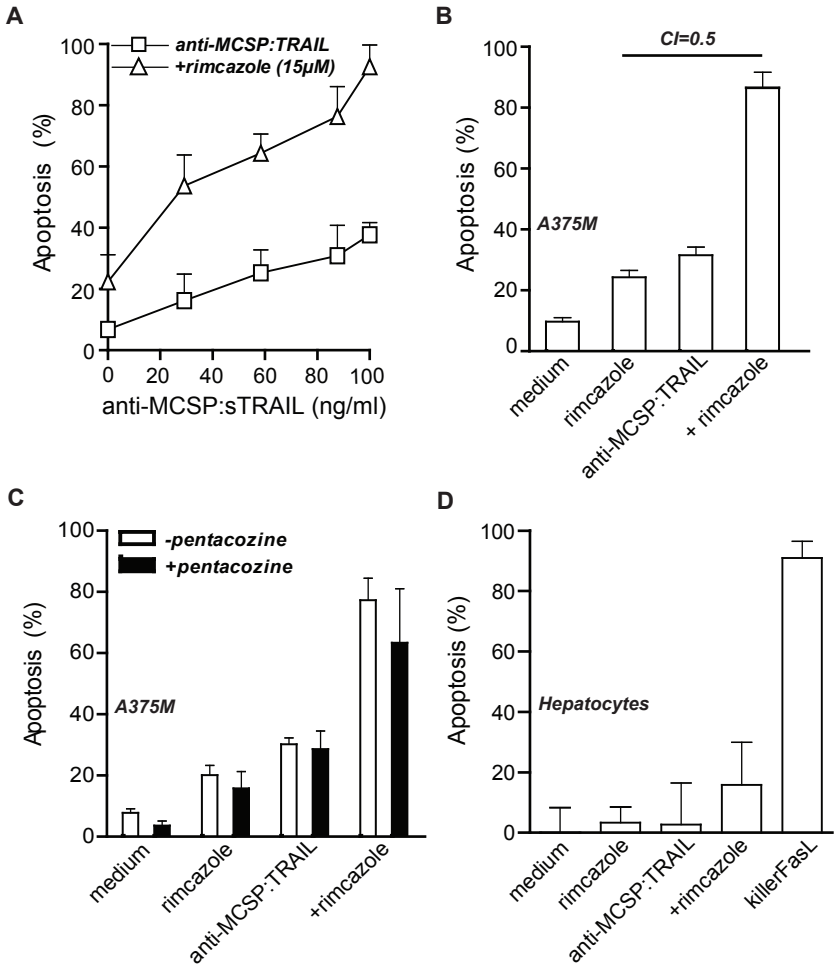

Figure 9. Synergistic induction of apoptosis by anti-MCSP:TRAIL and rimcazole A. A375M cells were treated with increasing concentrations of anti-MCSP:TRAIL in the presence or absence of rimcazole for 16h and apoptosis was assessed by Δψ. **B** A375M cells were treated with rimcazole (15 μM), anti-MCSP:TRAIL (100 ng/ml) or both for 16h, apoptosis was assessed by Δψ and the cooperativity index (CI) was calculated as indicated in the materials and methods section. **C** A375M cells were treated with rimcazole (15 μM), anti-MCSP:TRAIL (100 ng/mL) or both in the presence or absence of pentacozine (200 nM) for 16h and apoptosis was assessed by Δψ. **D** Hepatocytes were treated with, rimcazole (15 μM), anti-MCSP:TRAIL (100 ng/ml) or both for 16h and apoptosis was assessed by Δψ. KillerFasL (100 ng/ml) was used as a positive control for hepatocyte toxicity.

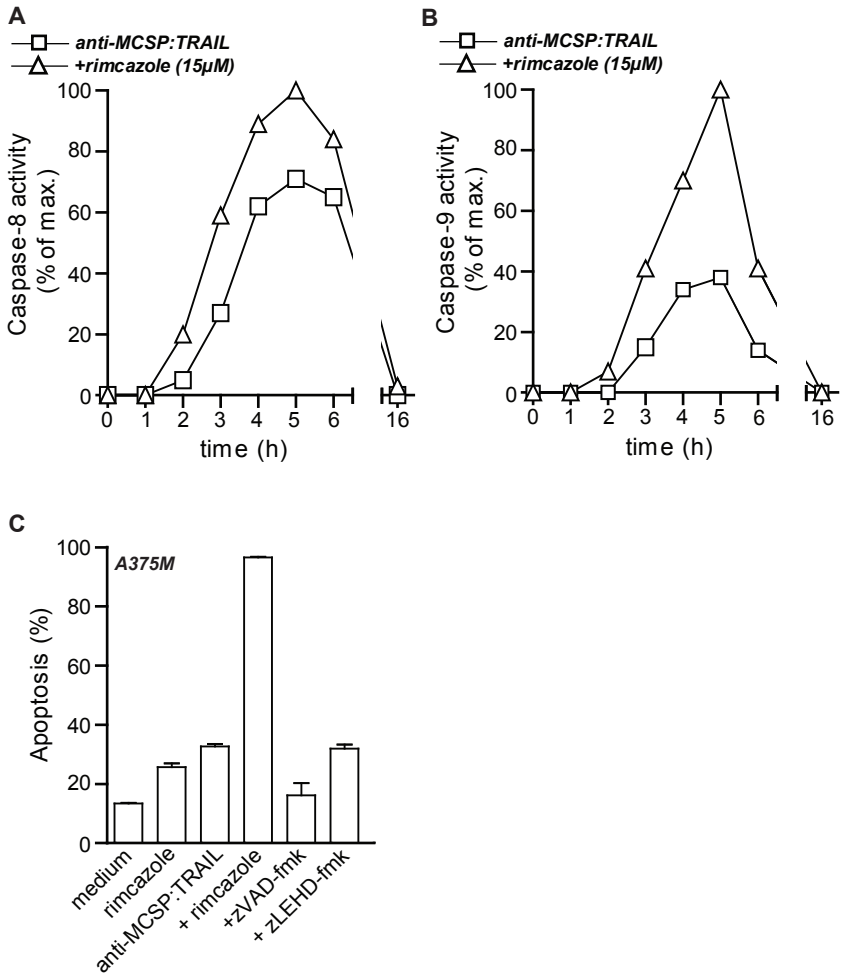

Figure 10. Synergy between rimcazole and anti-MCSP:TRAIL is caspase dependent. Activity of **A** Caspase-8, **B** Caspase-9 was assessed in A375M cells after incubation with anti-MCSP:TRAIL in the presence or absence of rimcazole (15 μM) for 1, 2, 3, 4, 5, 6 or 16h **C** A375M cells were treated for 16h with 100 ng/mL of anti-MCSP:TRAIL and/or rimcazole (15 μM) in the presence or absence of zVAD-FMK (20 μM) or zLEHD-FMK and apoptosis was assessed by $\Delta\psi$.

by promoting caspase-8 activation. Of note, anti-MCSP:TRAIL in combination with rimcazole lacked apoptotic activity towards hepatocytes (Fig. 9D), suggesting that this combination retains the favorable toxicity profile associated with TRAIL and rimcazole alone.

Conclusions

We provide evidence that anti-MCSP:TRAIL, a TRAIL fusion protein targeted to melano-

ma-expressed MCSP, inhibits MCSP tumorigenic signaling and simultaneously induces TRAIL apoptotic signaling. Consequently, fusion protein anti-MCSP:TRAIL potently inhibits outgrowth of melanoma cells both *in vitro* and *in vivo* and this effect can be further enhanced with the cytotoxic agent rimcazole. Based on the above, we postulate the following working model for anti-MCSP:TRAIL (see also Fig. 11B); anti-MCSP:TRAIL binds to MCSP and inhibits MCSP-signaling, which includes inhibition of src-kinases and FAK, the STAT transcription factors, and various anti-apoptotic modulators such as p53, TOR, JNK. Concurrently, interaction of the sTRAIL domain with its agonistic receptors TRAIL-R1 and TRAIL-R2 triggers potent induction of apoptosis. Notably, TRAIL/TRAIL-R interaction also triggers dephosphorylation of the STAT family, which may contribute to sensitization of cells to apoptosis [33,34]. Thus, melanoma cells are eliminated on the one hand by MCSP-mediated sensitization of melanoma cells to apoptosis and on the other hand by the concurrent activation of TRAIL-apoptotic signaling. Taken together, anti-MCSP:TRAIL is a novel immunotherapeutic agent that, either alone or in combination with rimcazole, may be of potential value for treatment of advanced melanoma.

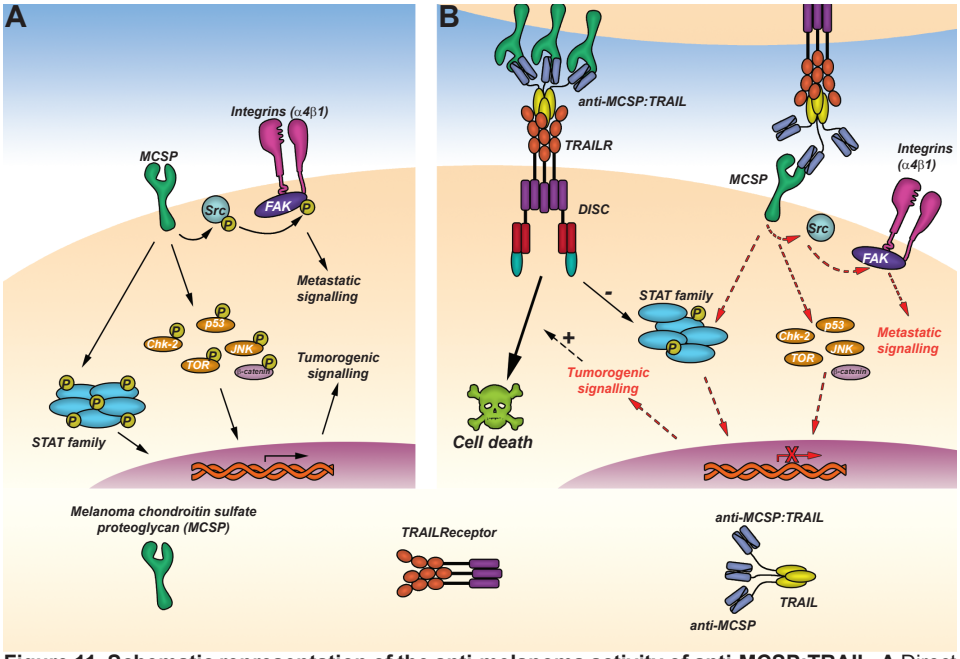

Figure 11. Schematic representation of the anti-melanoma activity of anti-MCSP:TRAIL. A Direct or indirect MCSP-mediated signaling involves (integrin-dependent) FAK and Src phosphorylation and maintains phosphorylation of downstream transcription factors and apoptotic modulators. MCSP thus contributes to pro-survival and metastatic signals. **B** Binding of anti-MCSP:TRAIL inhibits MCSP-mediated signaling and concomitant activation of pro-metastatic/-survival signals and thereby sensitizes cells to apoptosis induction via binding of the TRAIL moiety to TRAILR.

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Cell surface delivery of TRAIL strongly augments the tumoricidal activity of T-cells

Marco de Bruyn[1], Yunwei Wei[1,2], Valerie R. Wiersma[1], Douwe F. Samplonius[1], Harry G. Klip[3], Ate G.J. van der Zee[3], Baofeng Yang[4], Wijnand Helfrich[1] and Edwin Bremer[1]

[1]Department of Surgery, Surgical Research Laboratories, University Medical Center Groningen (UMCG), University of Groningen, Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.

[2]Department of General Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, 150001, China.

[3]Department of Gynecological Oncology, University Medical Center Groningen (UMCG), University of Groningen, Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.

[4]Department of Pharmacology, State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Harbin Medical University, Harbin, Heilongjiang 150081, China.

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Abstract

Adoptive T-cell therapy generally fails to induce meaningful anti-cancer responses in patients with solid tumors. Here, we present a novel strategy designed to selectively enhance the tumoricidal activity of T-cells by targeted delivery of TRAIL to the T-cell surface. We constructed, produced and purified two fusion proteins: anti-CD3:TRAIL and K12:TRAIL that selectively target TRAIL to the T-cell surface antigens CD3 and CD7, respectively. Tumoricidal activity of T-cells in the presence of these fusion proteins was assessed in solid tumor cell lines, primary patient-derived malignant cells and in a murine xenograft model. Both anti-CD3:TRAIL and K12:TRAIL increased the tumoricidal activity of T-cells towards cancer cell lines and primary patient-derived malignant cells >500-fold. Furthermore, T-cell surface delivery of TRAIL strongly inhibited tumor growth and increased survival time of xenografted mice >6-fold. In conclusion, targeted delivery of TRAIL to cell surface antigens of T-cells potently enhances the tumoricidal activity of T-cells. This approach may be generally applicable to enhance the efficacy of adoptive T-cell therapy.

Introduction

While efficacious in certain virus-mediated cancers, adoptive T-cell therapy generally fails to induce meaningful anti-cancer responses in patients with solid tumors. The cause for this lack of clinical efficacy is multi-factorial by nature and includes the intrinsic or acquired resistance of malignant cells to cytotoxic T-cell effector mechanisms such as granzyme/perforin-mediated lysis (1-5) and Fas-mediated killing (6;7). To overcome or circumvent this potential pitfall of adoptive T-cell therapy, we propose to selectively expand the T-cell armamentarium with additional tumoricidal effector molecules, in particular with the Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL).

TRAIL is a type II transmembrane protein normally expressed on NK cells, where it is essential for NK-cell-mediated tumor immune surveillance (8;9). In contrast, the expression of TRAIL on resting T-cells is typically very low (10;11). Nevertheless, a slight induction of TRAIL expression on T-cells, by ectopic expression of TRAIL or by *ex vivo* co-stimulation with anti-CD3 and IFN-α, already potently increases their tumoricidal activity (11;12). Furthermore, T-cell expressed TRAIL is essential for graft versus tumor (GVT) activity during allogeneic hematopoietic cell transplantation (AHCT) (10). Importantly, T-cell expressed TRAIL does not contribute to deleterious graft versus host disease (GVHD) activity (10). Taken together, these findings suggest that T-cells should gain additional tumoricidal potential when equipped with TRAIL at the cell surface.

Previously, we and others have reported an approach that may be particularly useful in this respect. In brief, recombinant soluble TRAIL (sTRAIL) was genetically linked to a tumor cell-selective antibody fragment (scFv). The resultant scFv:sTRAIL fusion protein was

produced, purified and subsequently used to selectively deliver TRAIL to the cell surface of tumor cells. Treatment of tumor cells expressing the relevant target antigen resulted in cell surface accretion of the scFv:sTRAIL fusion protein. Subsequently, tumor cell-bound TRAIL efficiently eliminated cancer cells (reviewed in chapter 2; ref (13)). Here, we utilized and adapted this approach to the selective delivery of high levels of TRAIL to the cell surface of T-cells, with the aim of expanding the cytotoxic armament of these T-cells with TRAIL (for schematic see Fig.1).

This approach was pre-clinically evaluated using two novel recombinant fusion proteins, designated anti-CD3:TRAIL and K12:TRAIL, that selectively bind to the T-cell surface antigen CD3 and CD7, respectively. Fusion protein anti-CD3:TRAIL contains a CD3-stimulatory antibody fragment, whereas K12:TRAIL contains a soluble form of the CD7-ligand K12. Anti-CD3:TRAIL and K12:TRAIL strongly potentiated the tumoricidal activity of T-cells on a panel of cancer cell lines, primary patient-derived malignant cells and in a murine xenograft model. This strategy may represent an easy to incorporate approach to enhance the efficacy of various forms of adoptive T-cell therapy in cancer.

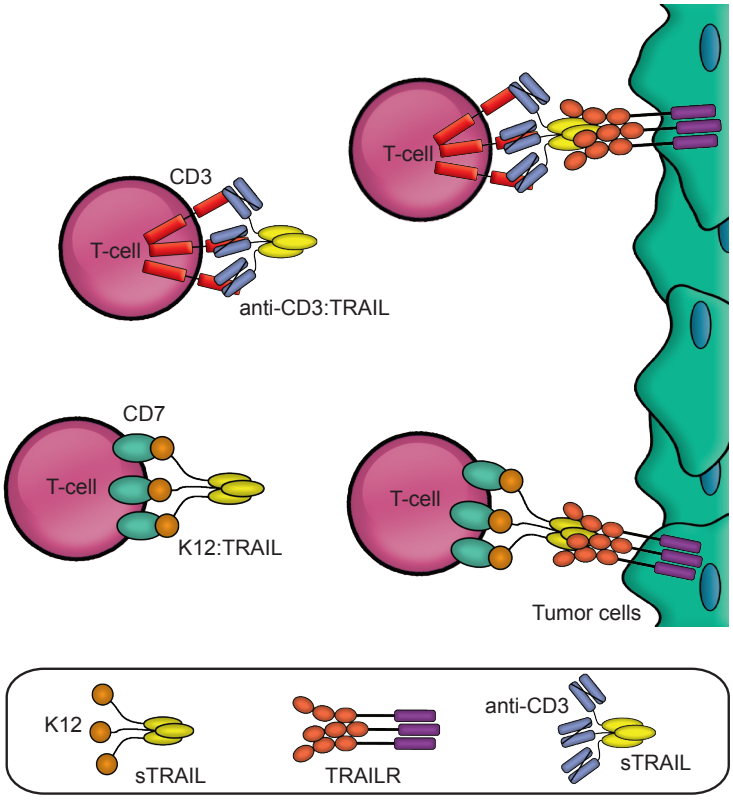

Figure 1. Cell surface delivery of TRAIL to augment the tumoricidal activity of T-cells. Fusion proteins K12:TRAIL and anti-CD3:TRAIL were designed to selectively bind to T-cell markers CD7 and CD3, respectively, and enhance levels of TRAIL on the surface of T-cells. Tumoricidal activity of T-cells should be augmented by virtue of TRAIL-TRAILR receptor mediated signaling in tumor cells.

Materials and Methods

Reagents

Anti-CD7 mAb TH-69 competes with K12:TRAIL for binding to CD7 and was a gift from Prof. Dr. Martin Gramatzki (University of Kiel, Germany). TRAIL-neutralizing mAb 2E5 was purchased from Kordia Life Sciences, FasL-neutralizing mAb Alf2.1 was purchased from Sigma-aldrich and TNF-α neutralizing fusion protein Enbrel was purchased from Wyeth. PE-labeled anti-TRAIL mAb B-S23 was purchased from Diaclone SAS and PE-labeled anti-CD69 mAb was purchased from IQ-products. Anti-TRAILR antibodies HS-201, HS-202, HS-203 and HS-204 and soluble TRAIL receptors TRAILR1-Fc, TRAILR2-Fc, TRAILR3-Fc and TRAILR4-Fc were purchased from Alexis. TRAIL ELISA was purchased from Diaclone and Cytokine ELISA was from SA Biosciences. DiI, DiO and FLUO-3-AM were purchased from Invitrogen. DioC6 was purchased from molecular probes. Actinomycin D, Valproic acid and Cyclohexamide were purchased from Sigma-Aldrich. Pan-caspase inhibitor zVAD-FMK, caspase-8 inhibitor zLEHD-fmk and caspase-9 inhibitor zIETD-fmk were purchased from Calbiochem. Anti-CD3 mAb and recombinant IL-2 were purchased from ImmunoTools. TNF-α was purchased from Abcam. FasL-Fc was a kind gift from Prof. Dr. Harald Wajant (University of Würzburg, Germany). RhTRAIL was purchased from R&D technologies.

Production of K12:TRAIL and anti-CD3:TRAIL

Fusion protein K12:TRAIL and anti-CD3:TRAIL were constructed by cloning the cDNA of soluble human K12 (aa 29-143) or the anti-CD3 antibody fragment scFvUCHT-1v9 in frame with human soluble TRAIL into previously described vector pEE14, yielding plasmids pEE14-K12:TRAIL and pEE14-anti-CD3:TRAIL, respectively. Fusion proteins K12:TRAIL and anti-CD3:TRAIL were produced in CHO-K1 cells essentially using previously described methods (14). Culture medium containing K12:TRAIL or anti-CD3:TRAIL was cleared by centrifugation (10.000 x g, 10 min), filter sterilized, and stored at -80°C until further use. Fusion proteins were purified via the N-terminal Hemagglutinin (HA)-tag using anti-HA affinity chromatography. Fusion protein anti-MCSP:TRAIL is a fusion protein that is essentially identical to anti-CD3:TRAIL except that it contains an anti-MCSP scFv instead of an anti-CD3 scFv. MCSP is expressed on cells of the melanocyte lineage, but not on T-cells or carcinoma cells.

Cell lines

SK-OV-3, OvCAR-3, A2780, HCT-8, HT29, A375M, A2058, SK-MEL-28, HEP3B, PLC-PRF-5, FADU, PC-3M, HK-2 and Ramos were obtained from the American Tissue Culture Collection (ATCC) and characterized by STR profiling, karyotyping and isoenzyme

analysis. A375M.FADD-DED cells were generated by transfection of parental A375M cells with a dominant negative FADD-DED construct using Fugene (Roche BV, Woerden, The Netherlands). A375M.FADD-DED cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 500 µg/ml Geneticin. HCT116-Luc was from Caliper Biosciences (Xenogen, Alameda, CA). NHDF-juv cells were from Promocell. Human umbilical vein endothelial cells (HUVEC) were isolated as described previously. Unless otherwise indicated, cells were cultured in standard RPMI (Cambrex Bio Science, Verviers, France) supplemented with 10% fetal calf serum.

Isolation of primary patient-derived tumor cells, T-cells and activation of T-cells
Experiments were approved by the local Medical Ethical Committee and patients/healthy volunteers signed for informed consent. Tumor tissues and ascitic fluids were obtained during surgery procedures and if necessary minced, after which adherent cells were cultured. Experiments were performed before passage number 5. Samples with a background apoptosis >30% were excluded from analysis. Normal fibroblast cultures originating from tumor tissue/ascitis were essentially established as described above for primary cancer cell cultures. Tumor infiltrating T-cells were isolated from the primary tumor cultures using Ammonium Chloride lysis. Peripheral blood lymphocytes (PBL) from blood of healthy donors were isolated using standard density gradient centrifugation (Lymphoprep, Axis-Shield PoC As., Oslo, Norway). Activated T cells were generated by culturing PBLs with anti-CD3 mAb (0.5 µg/mL; 72 h) and IL-2 (100 ng/mL; 48 h).

Analysis of fusion protein binding and TRAILR expression
The presence of cell surface-bound TRAIL was assessed by incubating cells with PE-conjugated anti-TRAIL mAb B-S23 (Diaclone SAS) for 60 min at 4°C followed by 3 washes. Cells were subsequently measured on an Accuri C6 flow cytometer. Cell surface display of TRAIL in time was determined by incubating cells with 50 pg/T-cell K12:TRAIL or anti-CD3:TRAIL for 60 minutes at 4°C followed by 3 washes in PBS. Cells were then incubated at 37°C for the time-points indicated and TRAIL expression was determined using mAb B-S23. The amount of K12:TRAIL or anti-CD3:TRAIL bound to an individual T-cell was determined using TRAIL ELISA. Briefly, $1x10^5$ T-cells were incubated with increasing concentrations of K12:TRAIL or anti-CD3:TRAIL for 1h. Subsequently, cells were centrifuged and supernatants used for TRAIL ELISA. Loss of K12:TRAIL and anti-CD3:TRAIL from the supernatant was calculated using the same concentration range of K12:TRAIL and anti-CD3:TRAIL in the absence of T-cells as a reference. Expression of TRAIL receptors was determined by incubating cells with mAb HS-201, HS-202, HS-203 or HS-204 for 60 min at 4°C, followed by 3 washes with PBS and incubation with PE-labeled Goat-anti-Mouse Ab. Cells were subsequently washed 3 more times with PBS and TRAILR expression was

determined using an Accuri C6 flow cytometer.

Analysis of cell death

For cell death assays, cancer cells were labeled with red fluorescent membrane label DiI (Invitrogen) in serum free medium for 15 min. followed by three washes in serum containing medium and subsequent culture in a 48-well plate ($3.0x10^4$cells/well). After 24 hours, cancer cells were treated for 16 hours with T-cells (activated or resting) at the indicated ratios in the presence or absence of K12:TRAIL (50 pg/T-cell), anti-CD3:TRAIL (50 pg/T-cell) or anti-MCSP:TRAIL (50 pg/T-cell) after which apoptosis was assessed in tumor cells by loss of mitochondrial membrane potential ($\Delta\Psi$) as previously described (14) using an Accuri C6 flow cytometer. Where indicated, caspase inhibitors were used at a final concentration of 10 μM. Actinomycin D was used at a final concentration of 2 μg/mL. Anti-TRAIL (2E5) and anti-FasL (Alf2.1) neutralizing mAbs and TNF-α neutralizing fusion protein Enbrel were used at a final concentration of 5μg/mL. For experiments involving Valproic acid (VPA), tumor cells were pre-treated for 24 h with 1 mM of VPA prior to addition of T-cells at the indicated ratios. For experiments involving concanamycin A, T-cells were treated with the indicated concentrations of concanamycin A for 2h prior to addition to tumor cells. Tumor cells were subsequently treated with these T-cells for 4h in the presence of concanamycin A after which induction of apoptosis was assessed. For outgrowth experiments using patient ascitic fluid, anti-CD3:TRAIL, anti-CD3 mAb or rhTRAIL were added at a final concentration of 100 ng/mL to a mixture of ascitic fluid and normal culture medium (50% v/v).

Time-lapse confocal microscopy

Tumor cells were labeled with membrane label DiO (green) by incubation of $1x10^6$ cells with 5 μL DiOI in serum free RPMI1640 for 5 min. at 37°C. Cells were then washed three times with RPMI1640+10% FCS and DiO-labeled tumor cells were cultured in a glass cover slide ($3.0x10^4$ cells/well). After 24 hours, T-cells labeled with DiI (red) using the same procedure were added to the tumor cells at the indicated Effector-to-Target (E:T) ratios. Time-lapse confocal microscopy was performed on a Solamere Nipkow Disk CLSM equipped with temperature/CO2 controlled cabinet and multi-cell track table.

Mechanical stimulation of CD3-signaling and Calcium Flux

Glass coverslides in petridishes were coated overnight with 10 μg/mL TRAILR1-Fc, TRAILR2-Fc, TRAILR3-Fc or TRAILR4-Fc. Coverslides were subsequently washed with PBS and blocked with normal human serum for 1h. T-cells were labeled with FLUO-3-AM by incubating the cells with 5 μM FLUO-3-AM ester in 100 μL PBS for 15 minutes at 20°C, followed by addition of 400 μL PBS with 10% FCS and 45 minutes of incubation at 37°C. Next, FLUO-3-AM labeled T-cells were added to the TRAIL-R1, -R2, -R3 or -R4 coated

glass slides. T-cells were mechanically stimulated by using a micropipette as previously described (15). Ca-flux was measured and quantified on a TILL iMIC fluorescence microscope equipped with temperature/CO_2 controlled cabinet. Ca-flux is depicted as the mean fluorescent intensity (MFI) at any given time point (MFIT) / the MFI at T=0.

Cytokine ELISA

T-cells were incubated with tumor cells for 16h at an effector-to-target ratio of 5:1 in the presence or absence of 50 pg/T-cell anti-CD3:TRAIL. Where indicated, mAb 2E5 was added to block TRAIL-TRAILR interaction. Alternatively, T-cells were treated with anti-CD3 mAb for 16h to elicit T-cell activation and cytokine release. Cell supernatants were harvested, centrifuged and used for cytokine ELISA according to the manufacturer's instructions.

HCT-116-luc xenograft mouse model

Experiments were approved by the Committee for Research and Animal Ethics of the UMCG. Male athymic mice (Harlan) were intra-peritonealy inoculated with HCT116-luc (1 x 10^6 cells). Animals were subsequently treated with a single i.p. dose of 5x10^6 T-cells and ~150 µg/Kg K12:TRAIL or anti-CD3:TRAIL as indicated. Tumor growth was monitored by luminescent imaging of mice from the ventral abdominal side using an IVIS Spectrum (Xenogen) optical imager. Animals were sacrificed by cervical dislocation when bioluminescent signal was increased 10-fold compared to day of inoculation. For localization of T-cells, T-cells were labeled with CellVue®NIR815 membrane labeling kit according to the manufacturer's instructions. Specific fluorescent signal was determined by subtracting the fluorescent signal from saline treated mice.

Statistical analysis

Data reported are mean values±SD of three independent experiments. Data was analyzed by one-way ANOVA followed by Tukey-Kramer post test or, where appropriate, by two-sided unpaired Student's t-test. Where indicated * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

Results

Tumoricidal activity of T-cells is potently augmented by delivery of TRAIL to the T-cell surface

In order to selectively deliver TRAIL to the cell surface of T-cells, the CD3-targeted fusion protein anti-CD3:TRAIL and the CD7-targeted fusion protein K12:TRAIL were generated. In line with literature, *ex vivo* activated T-cells obtained from healthy volunteers did not detectably express TRAIL (Fig.2A). Incubation of these TRAIL-negative T-cells with anti-CD3:TRAIL or K12:TRAIL lead to the display of high levels of TRAIL on the T-cell surface (Fig.2A). A subsequent TRAIL ELISA demonstrated that a single T-cell binds ~50 pg of

K12:TRAIL and/or ~10 pg of anti-CD3:TRAIL (data not shown). This T-cell binding by anti-CD3:TRAIL or K12:TRAIL was selectively blocked by anti-CD3 or anti-CD7 monoclonal antibody (mAb), respectively (data not shown) and did not induce reciprocal T-cell death (Fig. 2B).

Importantly, T-cell selective binding by anti-CD3:TRAIL and K12:TRAIL did potentiate the tumoricidal activity of activated T-cells towards cancer cells, with >90% apoptosis at an Effector-to-Target (E:T) ratio of 2:1 (Fig. 2C). Activated T-cells alone failed to induce apoptosis at these E:T ratios, with only ~40% apoptosis at an E:T ratio of 100:1 (Fig. 2C). Addition of an analogous TRAIL fusion protein that cannot bind T-cells (anti-MCSP:TRAIL; chapter 5) did not augment cell death in OvCAR-3 cells (Fig. 2D).

This enhanced tumoricidal activity towards OvCAR3 was dose-dependent (Fig. 3A and 3B), apparent within 2h and progressed to a maximum effect within 16h (Fig. 3C and Video S1 and S2). In addition, while the display of K12:TRAIL and anti-CD3:TRAIL on the T-cell surface was reduced over time (Fig. 3D and 3E, respectively), T-cells pre-incubated with excess K12:TRAIL or anti-CD3:TRAIL prior to addition to tumor cells (up to 48 h) fully retained their enhanced apoptotic activity (Fig. 3F). This indicates that in the presence of normal receptor turnover, sufficient K12:TRAIL or anti-CD3:TRAIL remain displayed on the T-cell surface. Importantly, the potentiating effect of anti-CD3:TRAIL and K12:TRAIL on the tumoricidal activity of T-cells was detected in a panel of 14 solid tumor cell lines (Fig. 4A) and in an extensive panel of short term cultures of primary patient-derived cancer cell samples (Fig. 4B; anti-CD3:TRAIL, Fig. 4C; K12:TRAIL). In these patient-derived malignant cell cultures, treatment with anti-CD3:TRAIL or K12:TRAIL and T-cells, completely disrupted the monolayer within 5h as exemplified for K12:TRAIL in Fig. 4D and Video S3 and S4. Consistent with other reports on TRAIL sensitivity, we found no correlation between TRAILR expression and sensitivity to K12:TRAIL (Fig. 5). However, there was a slight negative correlation between sensitivity to anti-CD3:TRAIL and expression of TRAILR4 (Fig. 5D), but not TRAILR1, R2 and R3 (Fig. 5A-C).

Taken together, anti-CD3:TRAIL and K12:TRAIL both potently augment the tumoricidal activity of activated T-cells toward cancer cell lines and patient-derived malignant cells by ~500-fold.

Anti-CD3:TRAIL induces tumoricidal activity by activation of resting T-cells

Anti-CD3:TRAIL comprises a T-cell stimulating CD3-antibody fragment. By virtue of this antibody fragment, anti-CD3:TRAIL might also be able to activate intrinsic cytotoxic effector mechanisms of T-cells. In line with this hypothesis, resting T-cells that lacked activity in co-culture experiments with OvCAR-3 gained potent cytotoxic activity in the presence of anti-CD3:TRAIL (Fig.6A, 100% apoptosis at E:T ratio 10:1). In contrast, resting T-cells remained ineffective in the presence of K12:TRAIL, with no cytotoxicity even at the highest E:T ratio

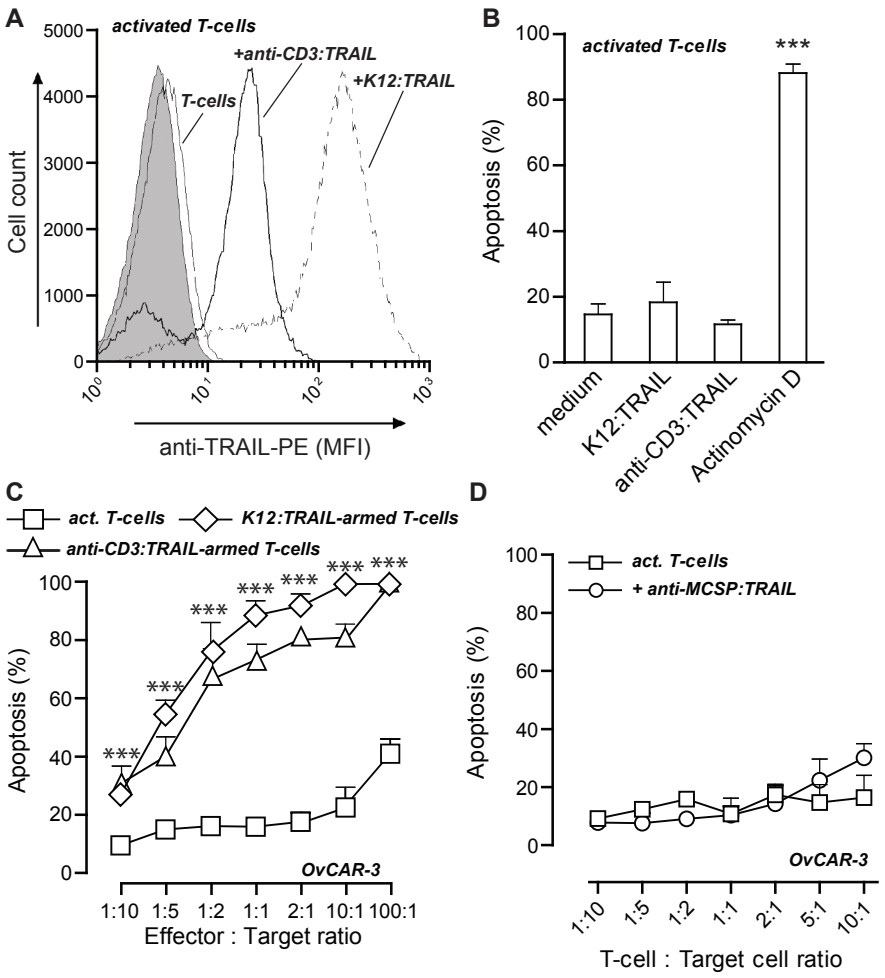

Figure 2. Tumoricidal activity of T-cells is potently augmented by delivery of TRAIL T-cell surface. A TRAIL expression on anti-CD3 + IL-2 activated T-cells was assessed by incubation of T-cells with PE-conjugated anti-TRAIL mAb (thin line). Cell surface TRAIL expression after binding of K12:TRAIL or anti-CD3:TRAIL was determined by pre-incubating T-cells with K12:TRAIL (hashed line) or anti-CD3:TRAIL (solid line). Shaded grey area represents fluorescence of PE-labeled isotype control. **B** Activated T-cells were incubated with OvCAR-3 cells in the presence or absence of K12:TRAIL (50 pg/T-cell) or anti-CD3:TRAIL (50 pg/T-cell) for 16h at the indicated E:T ratios and apoptosis was assessed in OvCAR-3 cells. **C** Anti-CD3 + IL-2 activated T-cells were treated with 50 pg/T-cell K12:TRAIL, 50 pg/T-cell anti-CD3:TRAIL or 2 µg/ml actinomycin D for 16h, after which cell death was quantified by flow cytometry. **D** Activated T-cells were incubated with OvCAR-3 cells in the presence or absence of anti-MCSP:TRAIL (50 pg/T-cell) for 16h at the indicated E:T ratios and apoptosis was assessed in OvCAR-3 cells.

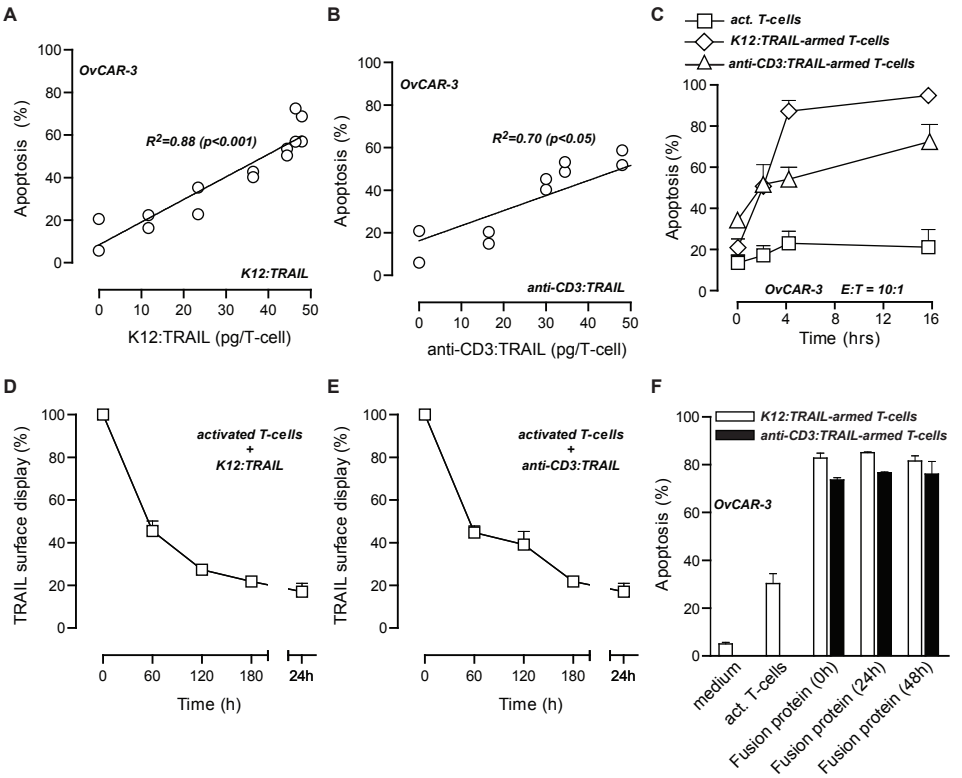

Figure 3. Tumoricidal activity of T-cells is potently augmented by delivery of TRAIL T-cell surface. A Activated T-cells were incubated with OvCAR-3 cells for 16h in the presence or absence of increasing concentrations of K12:TRAIL or **B** anti-CD3:TRAIL at an E:T ratio of 1:1 and apoptosis was assessed in OvCAR-3 cells. **C** Activated T-cells were incubated with OvCAR-3 cells in the presence or absence of K12:TRAIL (50 pg/T-cell) or anti-CD3:TRAIL (50 pg/T-cell) at an E:T ratio of 10:1 for the indicated time points and apoptosis was assessed in OvCAR-3 cells. **D** T-cells were incubated with K12:TRAIL (50 pg/T-cells) or **E** anti-CD3:TRAIL (50 pg/T-cell) for 1h and washed three times with PBS. TRAIL expression was subsequently determined at the indicated time-points using a PE-conjugated anti-TRAIL mAb. **F** T-cells were pre-incubated with K12:TRAIL (50 pg/T-cell) or anti-CD3:TRAIL (50 pg/T-cell) for 0, 24 or 48h prior to addition to tumor cells. Tumor cells were subsequently treated for 16h with these T-cells and apoptosis was assessed.

tested (Fig. 6A). Furthermore, anti-CD3:TRAIL consistently reduced long term outgrowth by >50% in ascitic fluid from ovarian cancer patients that contains both primary malignant cells and autologous T-cells (Fig. 6B and 6C). Neither an anti-CD3 stimulatory mAb nor an untargeted soluble rhTRAIL preparation alone inhibited cancer cell outgrowth (Fig. 6B).

In line with these activating properties, anti-CD3:TRAIL induced a rapid calcium-flux in T-cells co-cultured with OvCAR-3 cells (Fig. 7A) which was followed in time by an increase

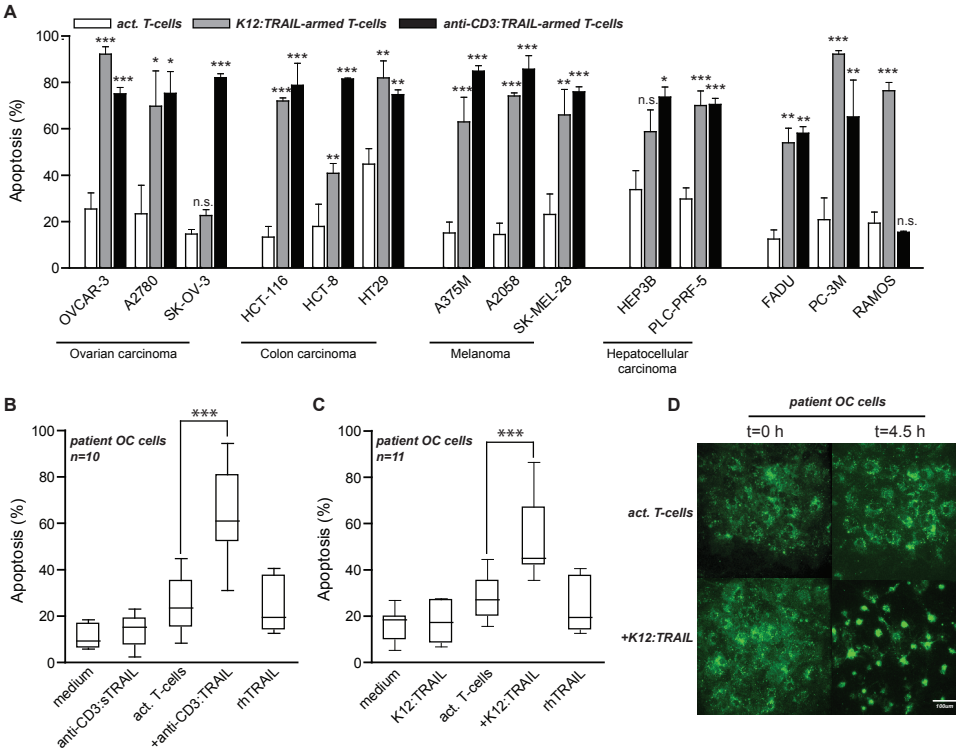

Figure 4. Tumoricidal activity of T-cells is potently augmented by delivery of TRAIL T-cell surface. A Cancer cell lines SK-OV-3, OvCAR-3, A2780, HCT-116, HCT-8, HT29, A375M, A2058, SK-MEL-28, HEP3B, PLC-PRF-5, FADU, PC-3M and RAMOS were treated for 16h with activated T-cells at an E:T ratio of 5:1 in the presence or absence of K12:TRAIL (50 pg/T-cell) or anti-CD3:TRAIL (50 pg/T-cell) and apoptosis was assessed. **B**, **C** Primary ovarian cancer cells were treated as indicated at an E:T ratio of 5:1 for 16h and apoptosis was assessed. **D** Primary tumor cells were fluorescently labeled using membrane label DiO (green) and incubated with DiI (red)-labeled activated T-cells in the presence or absence of K12:TRAIL (50 pg/T-cell) at an E:T ratio of 10:1. Subsequently, cells were used for live fluorescent imaging (5h) to observe morphological changes. For purpose of clarity, only green fluorescent cancer cells are depicted. See also video S1, S2, S3 and S4.

in the number of T-cells positive for the activation marker CD69 (Fig. 7B) and the release of pro-inflammatory T-cell cytokines (Fig. 7C). Interestingly, preventing cellular contact between T-cells and tumor cells abolished T-cell activation induced by anti-CD3:TRAIL (Fig. 7D), as did the addition of TRAIL neutralizing and TRAILR-blocking antibodies (Fig. 7B). In contrast, caspase inhibition did not block activation of anti-CD3:TRAIL-armed T-cells (Fig. 7B), but did block induction of apoptosis by rhTRAIL (data not shown). Further analysis using T-cells mechanically manipulated using a micropipet (Fig. 8A for schematic repre-

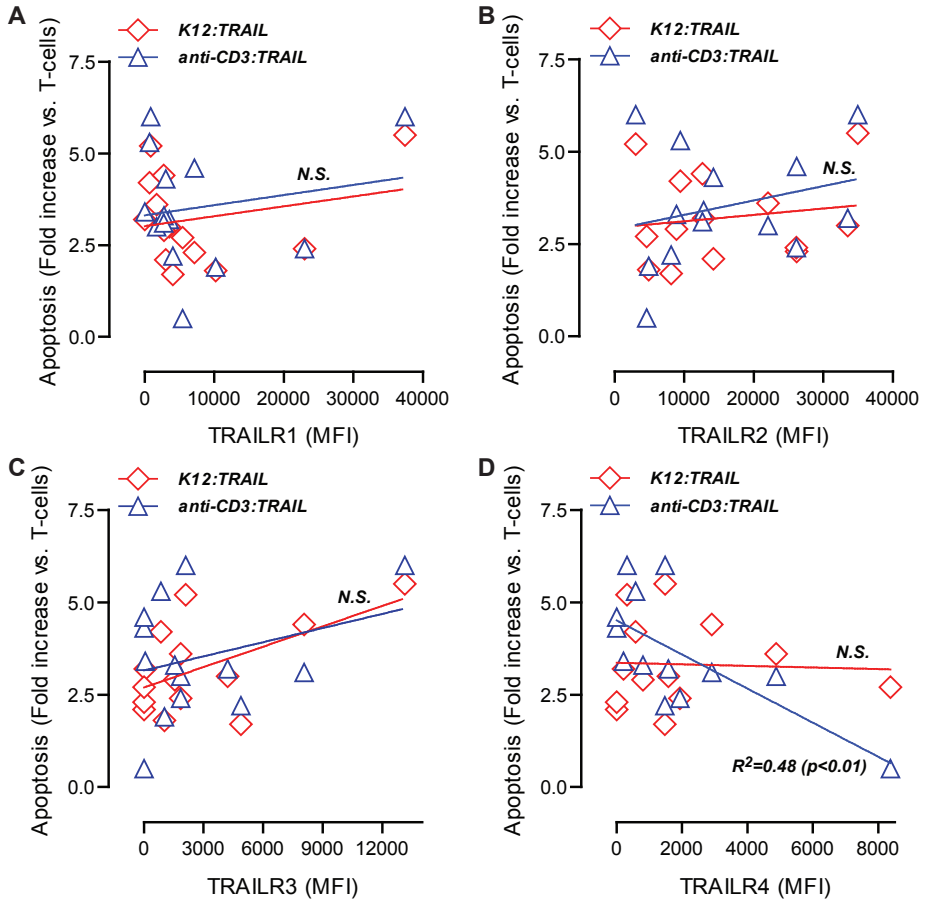

Figure 5. Tumoricidal activity of TRAIL-"armed" T-cells does not correlate with TRAILR expression levels. Expression of **A** TRAILR1 **B** TRAILR2 **C** TRAILR3 **D** TRAILR4 was determined on SK-OV-3, OvCAR-3, A2780, HCT-116, HCT-8, HT29, A375M, A2058, SK-MEL-28, HEP3B, PLC-PRF-5, FADU, PC-3M and RAMOS and plotted against the fold increase in apoptosis induced by T-cells armed with K12:TRAIL or anti-CD3:TRAIL.

sentation) indicated that anti-CD3:TRAIL-mediated T-cell activation was likely due to shear stress upon simultaneous binding of anti-CD3:TRAIL to CD3 on T-cells and TRAILR2 on tumor cells (Fig. 8A-C).

K12:TRAIL and anti-CD3:TRAIL enhance tumoricidal activity of resting T-cells via distinct mechanisms

Based on these activating properties, T-cell cytotoxicity induced by anti-CD3:TRAIL may at

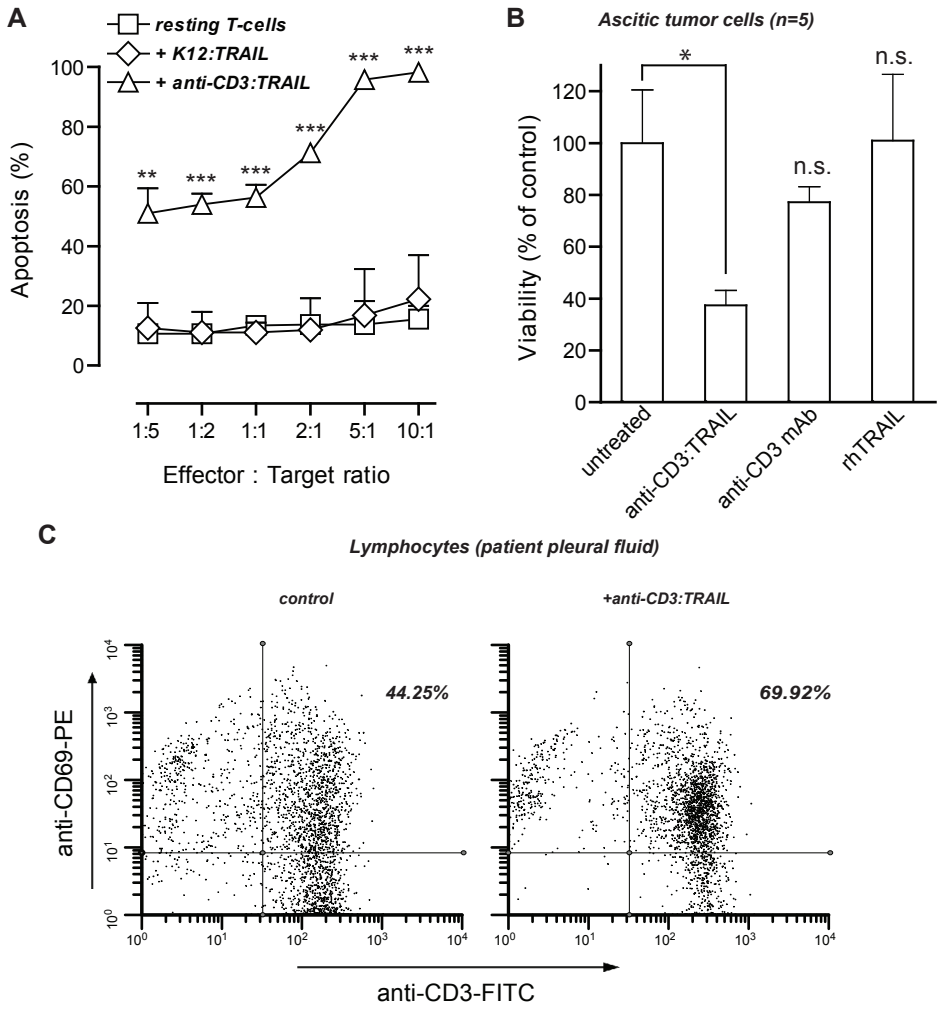

Figure 6. K12:TRAIL and anti-CD3:TRAIL enhance tumoricidal activity of resting T-cells via distinct mechanisms. A OvCAR-3 cells were treated with resting T-cells at the indicated E:T ratios for 48h in the presence or absence of K12:TRAIL (50 pg/T-cell) or anti-CD3:TRAIL (50 pg/T-cell) and apoptosis was assessed. **B** Ascitic fluid (n=5), was mixed with culture medium at a 1:1 ratio and treated with anti-CD3:TRAIL (50 pg/T-cell), anti-CD3 mAb (2 µg/mL) or rhTRAIL (100 ng/mL) for 12 days after which cell viability was assessed. **C** Tumor cells and Lymphocytes derived from OC patient ascitic fluid were treated for 16h with anti-CD3:TRAIL (50 pg/T-cell) and CD69 expression on lymphocytes was determined by flow cytometry using anti-CD3 as a T-cell marker.

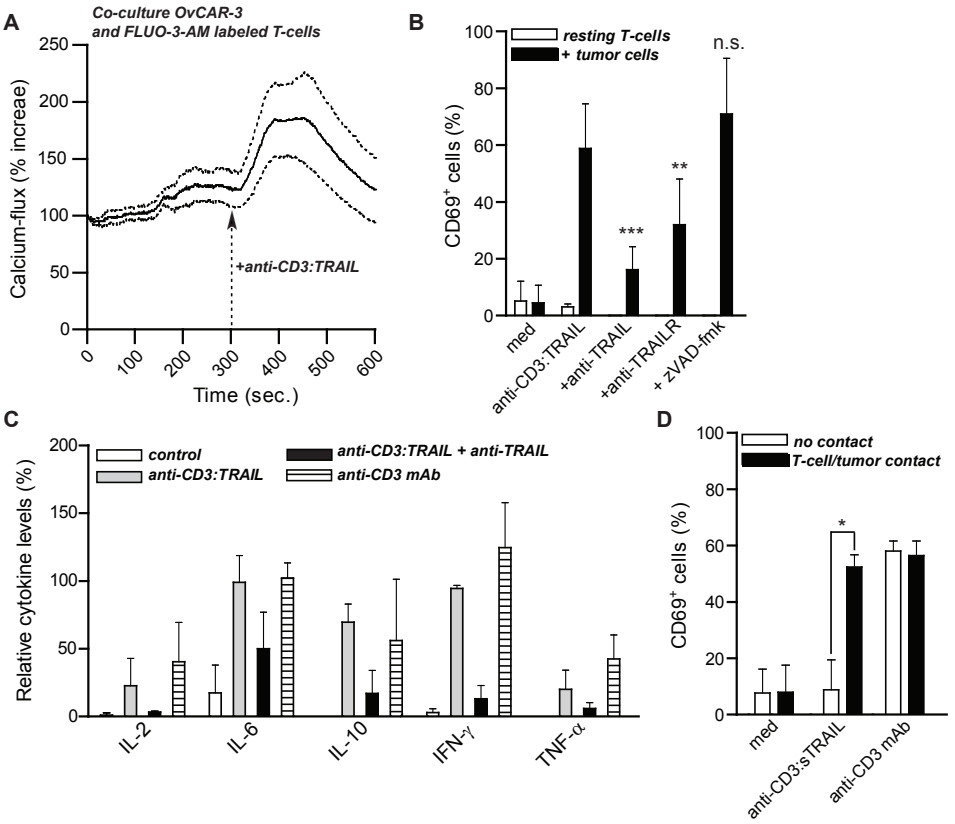

Figure 7. Anti-CD3:TRAIL induces activation of resting T-cells. A T-cells were labeled with FLUO-3-AM and added to OvCAR-3 cells grown on glass inserts in a Petri dish. Subsequently, anti-CD3:TRAIL (50 pg/T-cell) was added and Ca-flux was measured in T-cells (n=19). **B** Resting T-cells were incubated ± tumor cells and anti-CD3:TRAIL (50 pg/T-cell) in the presence or absence of TRAIL-neutralizing mAb 2E5, TRAILR blocking antibodies or pan-caspase inhibitor zVAD-fmk for 16h and CD69 expression on T-cells was assessed by flow cytometry. **C** Resting T-cells were co-incubated with tumor cells for 16h in the presence or absence of anti-CD3:TRAIL (50 pg/T-cell) or anti-CD3:TRAIL + anti-TRAIL mAb. Alternatively, T-cells were treated with anti-CD3 mAb (2 μg/mL). T-cell supernatants were harvested and relative cytokine levels in culture supernatant were determined by ELISA. **D** OvCAR-3 cells were coated in the top well of a trans-well system and resting T-cells were added to the top and bottom well. Subsequently, cells were treated with anti-CD3:TRAIL (50 pg/T-cell) or anti-CD3 mAb (2 μg/mL) for 16h and CD69 expression on T-cells was assessed.

least partly be attributable to stimulation of intrinsic T-cell cytotoxicity. Indeed, the potentiating effect on T-cell activity by anti-CD3:TRAIL, but not by K12:TRAIL, was dose-dependently blocked by the granzyme/perforin release inhibitor Concanamycin A (CMA) (Fig. 9A and 9B). In addition, A375M cells overexpressing a mutant form of the TRAILR adaptor

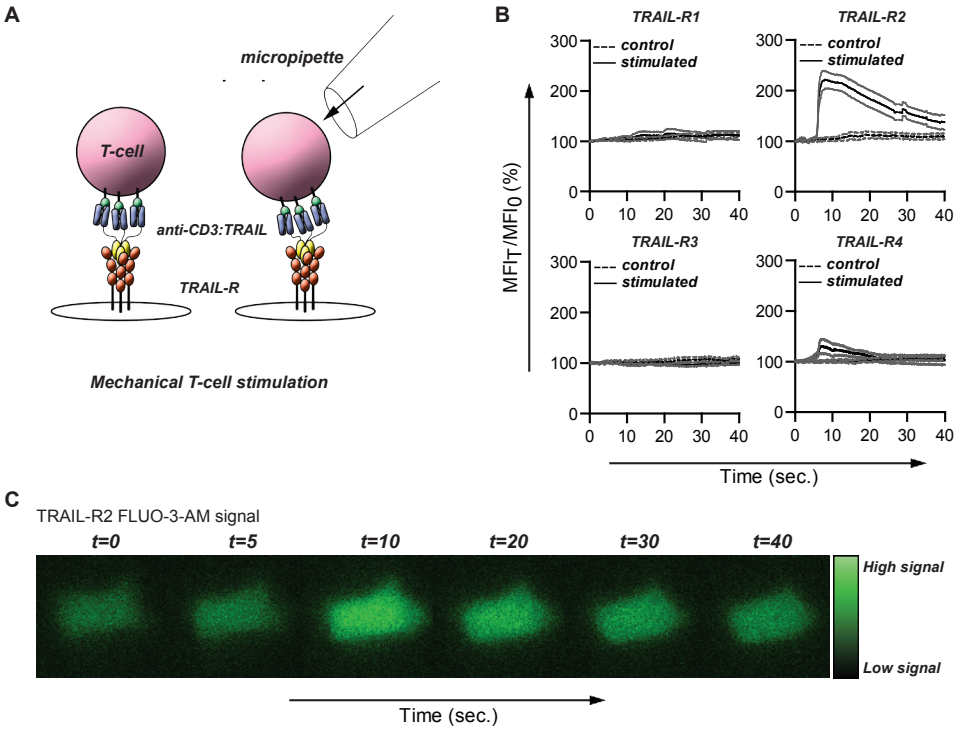

Figure 8. Anti-CD3:TRAIL can induce activation of resting T-cells via shear stress. A Schematic representation of mechanical CD3-mediated Ca-flux. In brief, FLUO-3-AM labeled T-cells are added to TRAILR-coated wells, after which a micropipette is used to induce mechanical shear stress and fluorescent intensity is monitored by microscopy. **B** Ca-Flux induced in anti-CD3:TRAIL-loaded T-cells on TRAIL-R1, -R2, -R3 or -R4 coated wells. **C** representative images of Ca-flux upon mechanical stimulation of anti-CD3:TRAIL-loaded T-cells on TRAILR2 coated wells.

protein FADD (FADD-DED) that inhibits TRAILR mediated caspase activation, remained sensitive to treatment with T-cells+anti-CD3:TRAIL, but not to T-cells + K12:TRAIL (Fig. 9C). Inhibition of other typical cytotoxic T-cell effectors, such as the tumoricidal proteins FasL and TNF-α, failed to inhibit apoptotic activity of anti-CD3:TRAIL/K12:TRAIL towards OvCAR-3 cells (Fig. 9D). Apoptotic activity of anti-CD3:TRAIL/K12:TRAIL-armed T-cells was abrogated by a pan-caspase inhibitor (Fig. 9D) and by inhibition of caspase-8 or caspase-9 (Fig. 9D). Of note, the potentiating effect of anti-CD3:TRAIL on tumoricidal T-cell activity closely resembled that of T-cells redirected with the bispecific antibody Bis-1 (anti-CD3:anti-EpCAM), which was also abrogated by CMA (Fig. 9E). Therefore, the mode of action of anti-CD3:TRAIL appears to be largely dependent on granzyme/perforin-mediated lysis, whereas K12:TRAIL functions via activation of initiator caspase-8/-9 and effector caspases. Furthermore, the inhibitory effect of caspase-9 blockade suggests that mitochon-

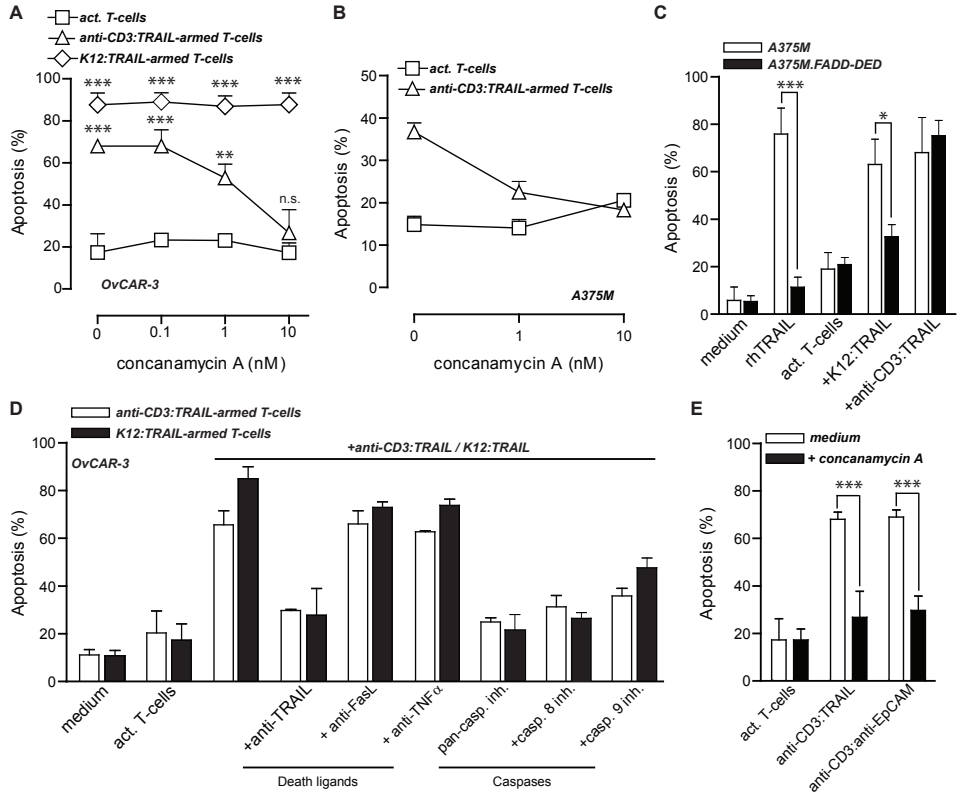

Figure 9. K12:TRAIL and anti-CD3:TRAIL enhance tumoricidal activity of resting T-cells via distinct mechanisms. A OvCAR-3 or **B** A375M cells were incubated for 4h at an E:T of 10:1 with activated T-cells ± K12:TRAIL (50 pg/T-cell) or anti-CD3:TRAIL (50 pg/T-cell) in the presence of the indicated concentrations of concanamycin A and apoptosis was assessed. **C** A375M or A375M.FADD-DED cells were treated rhTRAIL (100ng/mL), act-T-cells (5:1) ± K12:TRAIL (50 pg/T-cell) or anti-CD3:TRAIL (50 pg/T-cell) for 16h and apoptosis was assessed. **D** OvCAR-3 cells were treated for 16h with activated T-cells ± K12:TRAIL (50 pg/T-cell) or anti-CD3:TRAIL (50 pg/T-cell) at an E:T ratio of 10:1 in the presence or absence of TRAIL-neutralizing mAb 2E5, FasL-neutralizing mAb Alf2.1, TNF-α neutralizing fusion protein Enbrel, pan-caspase inhibitor zVAD-fmk, caspase-8 inhibitor zIETD-fmk or caspase-9 inhibitor zLEHD-fmk and apoptosis was assessed. **E** OvCAR-3 cells were treated with activated T-cells, anti-CD3:TRAIL-armed T-cells or activated T-cells redirected using bispecific antibody anti-CD3:anti-EpCAM in the presence or absence of 10 nM concanamycin A and apoptosis was assessed.

drial amplification of the caspase-8 apoptotic signal is required for effective tumoricidal activity.

K12:TRAIL and anti-CD3:TRAIL inhibit growth of colorectal carcinoma in vivo

The potent *in vitro* effects of K12:TRAIL and anti-CD3:TRAIL prompted us to evaluate activity against tumor cells *in vivo*. To this end, we xenografted colorectal carcinoma cell line HCT116-luc intraperitoneally in mice as a model for advanced metastatic colon carcinoma. HCT116-luc cells rapidly established tumor nodules throughout the abdominal cavity, resulting in a median survival of only 7 days (Fig. 10B and 10C). Importantly, growth of HCT116-luc in vivo was significantly suppressed by co-treatment with K12:TRAIL and activated T-cells, compared to treatment with either K12:TRAIL or T-cells alone. Indeed, median survival was increased 6-fold and one mouse survived for the duration of the experiment (70d) with minimal residual disease (Fig. 10A, 10B; 42d vs. 7d). Of note, co-treatment with K12:TRAIL and T-cells did not have obvious off-target toxicity in these mice, with no apparent weight loss (data not shown). In addition, fibroblasts (NHDF-juv), normal kidney cells (HK-2) and endothelial cells (HUVEC) were resistant to co-treatment with K12:TRAIL and T-cells (data not shown). Similarly, co-treatment of mice with T-cells and anti-CD3:TRAIL resulted in a striking increase in median survival with all treated mice remaining tumor free for the duration of the experiment (Fig. 10A and 10C). Treatment with anti-CD3:TRAIL alone had no effect on animal survival (Fig. 10C). Of note, while no deleterious effects on animal well being were observed during the experiment, treatment of NHDF-juv, HK-2 and HUVEC with anti-CD3:TRAIL *in vitro* did enhance T-cell-mediated toxicity towards these cells (data not shown). To determine whether T-cells remained localized to the intraperitoneal cavity, we used fluorescently labeled T-cells (Fig. 11). Treatment with activated T-cells alone had no effect on tumor load (3/3 mice), although T-cells did localize to tumor nodes in the abdominal cavity (Fig. 11). Treatment with activated T-cells+K12:TRAIL resulted in 1 tumor-free mouse after 2 days and 2 out of 3 tumor-free mice after 5 days. Interestingly, one of the mice still bearing tumor cells after 2 days displayed a focal co-localization of T- and tumor-cells (Fig.11) resulting in tumor cell eradication two days later (Fig. 11). T-cells+anti-CD3:TRAIL also co-localized to tumor sites (Fig. 11), but only 1 out of 3 mice were tumor free after 5 days. Taken together, K12:TRAIL and anti-CD3:TRAIL potently enhance anti-tumor activity of T-cells and inhibit growth of colorectal xenografts *in vivo*.

K12:TRAIL and anti-CD3:TRAIL are compatible with experimental strategies to ameliorate GvHD and optimize GvT

Earlier studies have shown that TRAIL-expression on T-cells is required for optimal graft-vs-tumor (GVT) activity during allogeneic hematopoietic cell transplantation (AHCT), but does not exacerbate graft-vs.-host disease (GVHD). Therefore, it was examined whether K12:TRAIL could be used in conjunction with therapies currently being evaluated for optimizing AHCT. In particular, the so-called Histone deacytelase inhibitors (HDACi) inhibit GVHD in preclinical settings (16;17), but are also well known to synergize with the pro-apoptotic activity of TRAIL (18;19). Of note, HDACi do not potentiate the GvT effect. In line

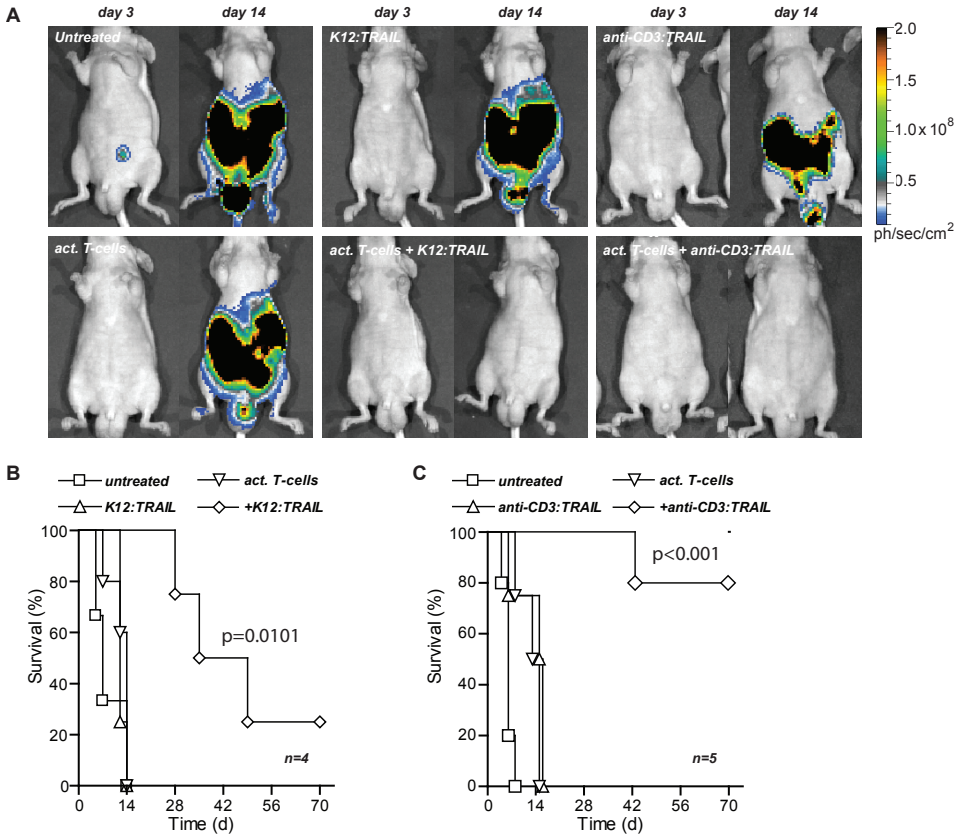

Figure 10. K12:TRAIL and anti-CD3:TRAIL inhibit growth of colorectal carcinoma in vivo. A Male athymic mice were injected i.p. with 1 x 10^6 HCT-116-luc cells followed by i.p. injection with either saline, activated T-cells (5 x 10^6), K12:TRAIL (0.25 mg/kg), anti-CD3:TRAIL (0.25 mg/kg), activated T-cells+K12:TRAIL or activated T-cells + anti-CD3:TRAIL and tumor growth was monitored by bioluminescence. Mice were sacrificed when tumor size reached a ten-fold increase compared to initial tumor load. Representative images of mice from all treatment groups on day 3 and day 14 after tumor cell injection are depicted. **B** Mice survival following treatment with act. T-cells, K12:TRAIL or act. T-cells + K12:TRAIL, as determined by a ten-fold increase in tumor size compared to start of treatment, was plotted in a Kaplan-Meier curve. **C** Mice survival following treatment with act. T-cells, anti-CD3:TRAIL or act. T-cells + anti-CD3:TRAIL, as determined by a ten-fold increase in tumor size compared to start of treatment, was plotted in a Kaplan-Meier curve. See also Video S5 and S6.

with this, pre-treatment with HDACi valproic acid (VPA) did not enhance the tumoricidal activity of T-cells alone (Fig.12A). In contrast, pre-treatment of OvCAR-3 cells with Valproic Acid (VPA) strongly optimized the potentiating effect of K12:TRAIL and anti-CD3:TRAIL on tumoricidal T-cell activity, with ~30% and ~50% cell death in OvCAR-3 at E:T ratio of 1:50,

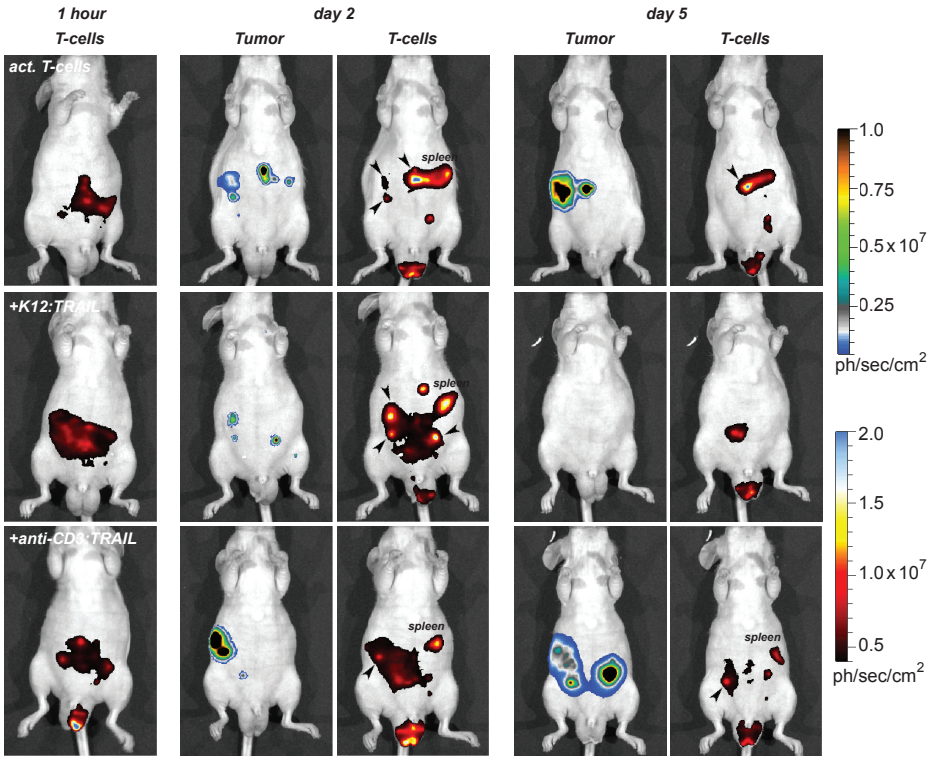

Figure 11. K12:TRAIL and anti-CD3:TRAIL inhibit growth of colorectal carcinoma in vivo. Male athymic mice were injected i.p. with 1 x 10^6 HCT-116-luc cells followed by i.p. injection with either saline, activated T-cells (1 x 10^6), activated T-cells+K12:TRAIL (0.25 mg/kg) or activated T-cells+anti-CD3:TRAIL (0.25 mg/kg). T-cell localization was subsequently monitored at the indicated time-points using fluorescence together with tumor localization using bioluminescence.

respectively (Fig. 12A-C). Interestingly, VPA enhanced the anti-tumor effect of K12:TRAIL, but not anti-CD3:TRAIL, towards two patient-derived tumor samples (Fig. 12D).

Various other strategies have been developed to selectively enhance GVT or ameliorate GVHD without affecting GVT. These strategies include the selective depletion of a population of alloreactive $CD69^{high}$ T-cells before infusion (20), or selectively transferring only $CD4^+$, $CD8^+$ or $CD25^+$ cells (21). K12:TRAIL and anti-CD3:TRAIL potentiated the tumoricidal activity of isolated $CD69^{low}$, $CD69^{medium}$, or $CD69^{high}$ T-cell populations to a similar degree (Fig. 12F). Similarly, K12:TRAIL and anti-CD3:TRAIL potentiated the tumoricidal activity of sorted $CD4^+$ and $CD8^+$ T-cells to a level similar to that observed in non-sorted T-cells (Fig. 12G). Therefore, K12:TRAIL and anti-CD3:TRAIL appear suitable for integration in existing strategies for optimizing AHCT.

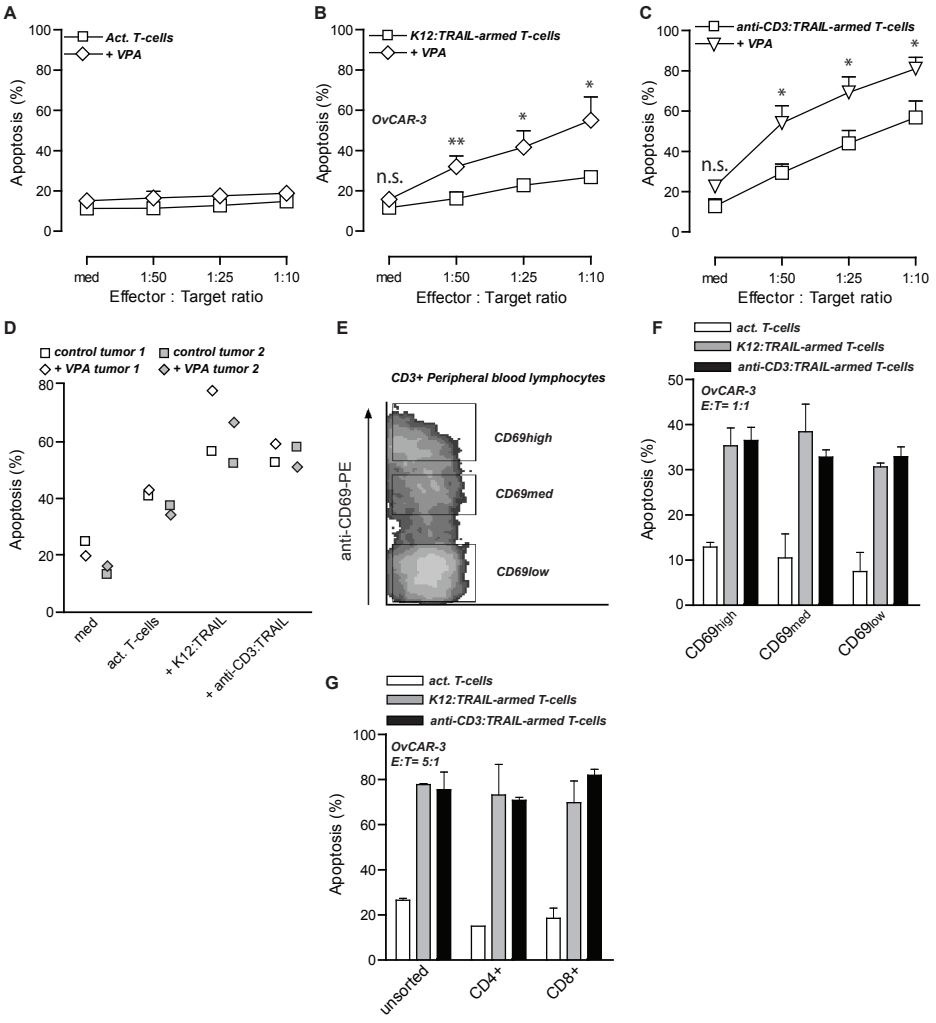

Figure 12. K12:TRAIL and anti-CD3:TRAIL are compatible with experimental strategies to ameliorate GvHD and optimize GvT. OvCAR-3 cells were pre-treated with VPA (1 nM) for 1day and subsequently treated for 16h with **A** activated T-cells **B** K12:TRAIL-armed T-cells (50 pg/T-cell) or **C** anti-CD3:TRAIL-armed T-cells (50 pg/T-cell) at the indicated E:T ratios. **D** Primary cancer cells (n=2) were pre-treated with VPA (1 nM) for 1day and subsequently treated for 16h with activated T-cells or K12:TRAIL/anti-CD3:TRAIL-armed T-cells (50 pg/T-cell) at an E:T ratio of 5:1. **E** Activated T-cells were double-stained for CD3 and CD69, whereupon $CD69^{low}$, $CD69^{medium}$ and $CD69^{high}$ populations were sorted from the CD3-positive cells. **F** OvCAR-3 cells were treated with $CD69^{low}$, $CD69^{medium}$ and $CD69^{high}$ in the presence or abscence of K12:TRAIL/anti-CD3:TRAIL (50 pg/T-cell) for 16h at an E:T ratio of 1:1. **G** OvCAR-3 cells were treated with $CD4^{+}$ or $CD8^{+}$ T-cell populations and K12:TRAIL/anti-CD3:TRAIL-loaded populations (50 pg/T-cell) for 16h at an E:T ratio of 5:1.

Discussion

Here we report on the preclinical evaluation of a new strategy designed to deliver high levels of the pro-apoptotic effector molecule TRAIL to the surface of T-cells and thereby safely augment anti-tumor T-cell activity. Arming T-cells with anti-CD3:TRAIL or K12:TRAIL resulted in an >500-fold increase in tumoricidal activity towards both cancer cell lines and primary patient-derived malignant cells. In addition, co-treatment with K12:TRAIL and T-cells significantly prolonged survival time of xenografted mice.

K12:TRAIL enhanced the tumoricidal activity of T-cells by activation of pro-apoptotic TRAIL-receptor signaling in tumor cells. Surprisingly, the potentiating activity of anti-CD3:TRAIL was found to be predominantly due to granzyme/perforin signaling, analogous to the activity of T-cell redirecting bispecific antibodies. In line with this mechanism of redirected lysis anti-CD3:TRAIL triggered CD3-mediated Ca-flux due to mechanical stress acting on the anti-CD3:TRAIL-CD3 complex. These findings corroborate with a recent report that suggest that CD3 in the T-cell Receptor Complex functions as a mechanoreceptor translating shear stresses into signaling (15;22). In this way, the function of anti-CD3:TRAIL appears analogous to artificial antigen-presenting complexes or native MHC-molecules. In view of these activating properties, anti-CD3:TRAIL may be particularly suited for e.g. *ex vivo* tumor cell purging of bone marrow. In addition, anti-CD3:TRAIL may be used in patients that suffer from malignant disease that is restricted to an anatomically confined region. Of interest in this respect are peritonitis carcinomatosis patients that undergo Heated Intra-Peritoneal Chemotherapy (HIPEC), an operation in which anti-CD3:TRAIL may easily be incorporated.

Applicability of K12:TRAIL is likely not limited to a specific kind of T-cell immunotherapy, but may be applied to e.g. *ex vivo* expanded tumor infiltrating lymphocytes, engineered T-cells, T-cell bodies, as well as in Graft-versus-Tumor (GVT) activity during allogeneic hematopoietic cell transplantation (AHCT). Indeed, perhaps the most obvious application of T-cell specific delivery of TRAIL using K12:TRAIL would be in the context of AHCT. Alloreactive donor T-cells are the primary mediators of GVT activity as well as graft-versus-host disease (GVHD). Earlier studies have shown that TRAIL-expression on T-cells was required for optimal GVT, whereas TRAIL did not contribute to GVHD (10). Although not investigated here, the data of the current study suggest that targeted delivery of TRAIL to donor T-cells using K12:TRAIL may be of value to selectively enhance GVT. Interestingly, novel GVHD-inhibitors like Histone deacytelase inhibitors (HDACi) (16;17) or rapamycin (23) are also known to sensitize cancer cells to TRAIL-induced apoptosis (18;19). In line with this, our experiments demonstrate further optimization of the potentiating effect of K12:TRAIL by pre-treatment of tumor cells with the HDACi Valproic Acid. Thus, rationally designed combinatorial strategies that include K12:TRAIL may well yield optimal GvT activ-

ity with no/minimal GvHD.

The here described immunotherapeutic approach to optimize T-cell immunotherapy may be further explored using TRAIL fusion proteins that incorporate other immunomodulatory scFvs or ligands that would simultaneously provide stimulatory signals to T-cells and add TRAIL signaling to the T-cell armamentarium. Likely combinations to this effect include stimulatory anti-CD28 scFvs (24) or inhibitory scFvs directed against Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) (25). In this respect, targeted activation of CTLA-4 signaling using a fusion protein CTLA-4:TRAIL fusion protein has been shown to limit T-cell activity and ameliorate autoimmunity in preclinical models (26). Reversely, inhibition of CTLA-4 signaling may potentiate anti-tumor T-cell activity.

In conclusion, cell surface delivery of TRAIL to T-cells potently augments the anti-tumor activity of T-cells *in vitro* and *in vivo* and might be widely applicable in current/experimental T-cell-based strategies to optimize anti-cancer efficacy.

Acknowledgements

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Treatment with TRAIL induces recruitment of the pro-phagocytic molecule Calreticulin to the Death Inducing Signalling Complex

Marco de Bruyn[1], Wijnand Helfrich[1], Valerie R. Wiersma[1], Douwe Samplonius[1], Paul Eggleton[2] and Edwin Bremer[1]

[1]Department of Surgery, Surgical Research Laboratories, University Medical Center Groningen (UMCG), University of Groningen, Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.

[2]Peninsula Medical School, University of Exeter, Exeter, UK.

Submitted

Abstract

Calreticulin is an apoptotic recognition phagocytic receptor that plays an important role in both autoimmune disease and cancer. Interestingly, previously calreticulin was shown to directly interact with TNF-family members FasL and TRAIL in an in vitro immunoassay. Furthermore, we have demonstrated that the interaction of calreticulin with FasL inhibits FasL-induced apoptosis in T-cells. Here, we investigated calreticulin - TRAIL/TRAIL-receptors interaction on cancer cells and the effect this association had on TRAIL-induced apoptosis in tumor cells. Our results indicate that calreticulin is recruited with TRAIL to the Death Inducing Signaling Complex of the TRAIL-Receptor, but does not inhibit or augment TRAIL-induced apoptosis. Of note, the interaction of calreticulin and TRAIL is time-dependent, peaks after ~30-60 minutes of TRAIL treatment and then subsides again. Interestingly, whereas calreticulin was associated with the anti-phagocytic receptor CD47 in control conditions, treatment with TRAIL triggered dissociation of calreticulin from CD47. Taken together, these data suggest that treatment of tumor cells with TRAIL leads to the recruitment of calreticulin towards the TRAIL apoptotic complex. Further elucidation of the role of calreticulin in TRAIL-signaling may help to pinpoint the immunological mechanisms of apoptotic cell recognition and removal upon TRAIL treatment.

Introduction

The endoplasmic reticulum chaperone calreticulin (CRT) is a pro-phagocytic ligand with roles in autoimmune disease and cancer (1). Normally, CRT is retained in the lumen of the endoplasmic reticulum (ER) (2;3), but CRT can also be excreted or displayed on the cell surface in association with other ER proteins (4-6). Interestingly, surface and/or soluble CRT appears to modulate signaling by TNF-family members and/or their receptors. For instance, binding of N-acetylmuramyl-L-alanyl-D-isoglutamine (L,D-MDP) to cell surface CRT (csCRT) induces activation of the pro-apoptotic TNFR1 pathway in RK13 cells (7). In contrast, soluble CRT (sCRT) inhibits apoptosis induced by Fas-ligand (FasL) in T-cells (8). This inhibition of FasL-mediated apoptosis in T-cells was a result of direct interaction between CRT and FasL (8). Interestingly, CRT was reported to not only bind to FasL, but also to the TNF-related apoptosis inducing ligand (TRAIL) in an ELISA type assay (9). The biochemical implications of CRT interaction with TNF-family members that are associated with regulating apoptosis have not been fully elucidated, but CRT may interact with these cell surface cell death regulators in different ways.

TRAIL is a member of the TNF family with pro-apoptotic activity towards malignant cells and little to no activity towards untransformed cells. As such, TRAIL has attracted

considerable interest for the treatment of cancer. TRAIL induces apoptosis via two agonistic receptors, TRAILR1 and TRAILR2, that both transmit apoptotic signals via the so-called Death Inducing Signaling Complex (DISC). The DISC contains the adaptor molecule Fas-Associated protein with Death Domain (FADD) and the pro-form of initiator caspase-8, and assembly of this complex leads to proximity-induced activation of the latter (10). Concomitantly, an intracellular proteolytic caspase cascade ensues that ultimately leads to the execution of apoptosis (reviewed in (11;12)).

Pro-apoptotic signaling via TRAILR1 and TRAILR2 may be competitively inhibited by two cell-surface expressed decoy receptors, TRAILR3 and TRAILR4. TRAILR3 is a phospholipid-anchored receptor that lacks a cytoplasmic domain (13), whereas TRAILR4 only retains a truncated intracellular domain that is incapable of apoptotic signaling (14). Therefore, both receptors are thought to function as decoy receptors for TRAIL (reviewed in (15)). Apoptotic signaling of the DISC can also be inhibited by recruitment of additional components, such as cFLIP isoforms (16). These proteins are structurally related to caspase-8, lack enzymatic activity and inhibit DISC-associated activation and processing of caspase-8 (for review see (17)).

Given the recent evidence that cell surface associated CRT can act as a cell death associated molecule and appears to enhance tumor recognition by the immune system (for review see (18)), we evaluated whether calreticulin might play a role in TRAIL-mediated apoptotic cell death of malignant cells. As cell surface CRT has previously been detected in melanoma cells (19) we focused on CRT interaction with TRAIL on the cell surface of melanoma cells using biochemical analyses. In addition, we evaluated whether calreticulin modulated TRAIL-induced apoptosis in cancer cells.

Material and methods

Reagents

Anti-CRT mAbs (Ab2907, Ab39818, Ab4 and Ab22684 [FMC 75]) were purchased from Abcam. CRT competing peptide Ab39895 is the peptide fragment used to generate Ab39818 in mice and was purchased from Abcam. Anti-CD47 antibody (B6H12.2) was purchased from Abcam. Anti-TRAIL mAb (B-S23) was purchased from Diaclone SAS. Anti-TRAILR2 mAb (HS-202) was purchased from Alexis. Chicken-anti-mouse-AlexaFluor596, Goat-anti-rabbit-AlexaFluor488 and Goat-anti-mouse-AlexaFluor488 were purchased from Invitrogen. PE-labeled anti-TRAIL mAb (B-S23) was purchased from Diaclone SAS. Anti-HA-Peroxidase mAb (3F10) and anti-HA sepharose beads (3F10) were purchased from Roche. Anti-FADD mAb (2782S) and anti-caspase8 mAb (18C8) were purchased from Cell signaling. Protein G-sepharose beads were purchased from GE Healthcare. CellEvent Caspase-3/-7 detection reagent was purchased from Invitrogen. Duolink proximity ligation assay was purchased from Olink Bioscience. Protease inhibitor cocktail was purchased

from Sigma-Aldrich. Soluble FasL was purchased from Enzo Life Sciences. Native human calreticulin was purified from Jurkat cells using a method as previously described (8). RhTRAIL was purchased from R&D systems.

Production of HA-TRAIL

HA-TRAIL was constructed by cloning the cDNA of HA-tagged TRAIL in frame into previously described vector pEE14, yielding plasmid pEE14-HA-TRAIL. HA-TRAIL was produced in CHO-K1 cells essentially using previously described methods (20;21). Culture medium containing HA-TRAIL was cleared by centrifugation (10.000 g, 10 min), filter sterilized, and stored at -80°C until further use. HA-TRAIL was purified via the N-terminal Hemagglutinin (HA)-tag using anti-HA affinity chromatography.

Cell lines

A375M was obtained from the American Tissue Culture Collection (ATCC) and characterized by short tandem repeat profiling, karyotyping and isoenzyme analysis. A375M.FADD-DED cells were generated by transfection of parental A375M cells using Fugene (Roche BV, Woerden, The Netherlands). Ramos, Ramos.cFLIP$_L$, Ramos.cFLIP$_S$, Ramos.FERM and Ramos.Ezrin were a kind gift from Dr. B.J. Kroesen (UMCG, the Netherlands). A375M and Ramos were cultured in RPMI1640 medium supplemented with 10% fetal calf serum. A375M.FADD-DED, Ramos.cFLIP$_L$, Ramos.cFLIP$_S$, Ramos.FERM and Ramos.Ezrin cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 500 µg/ml Geneticin.

Analysis of TRAIL binding to calreticulin

Native calreticulin (1 µg in 100 µL; 21 µM) in NaCO3 buffer (pH 9.6) was added to each well of a 96-well Nunc Maxisorp plate (Fisher Scientific). The plate was incubated overnight at 4°C and then washed with PBST. Aspecific binding was blocked with 3% w/v bovine serum albumin (BSA) in PBST for 1 hour at 37°C. Plates were then washed as described above. Increasing concentrations of rhTRAIL were then diluted in 3% w/v BSA in PBST and added to triplicate wells and incubated for 1 hour at 37°C. The plates were again washed as described above. Binding of TRAIL to calreticulin was detected by adding PE-conjugated anti-TRAIL antibody and incubating the plate for 1 hour at 4°C. The wells were washed as described before and the plates were read on an ELISA plate reader at 565 nm.

Microscopy

A375M melanoma cells were treated with rhTRAIL (100ng/mL; 5.1 nM) for 1h at 37°C, washed three times with PBS and fixed using 4% v/v PFA. Cells were subsequently washed three times with PBS, supplemented with anti-CRT or anti-TRAILR2 mAbs and incubated at

4°C for 1h. Cells were then washed three times with PBS, probed with secondary antibody and DAPI at 4°C for another 1h and washed three more times with PBS prior to examination. Confocal microscopy was performed on a Solamere Nipkow Disk CLSM equipped with temperature/CO2 controlled cabinet and multi-cell track table.

Proximity ligation assay

To determine direct CRT – TRAIL protein interaction, a proximity ligase assay (PLA) was employed. Aliquots of A375M cells, 4.5 x 10^3 in each chamber were cultured in permanox chamber slides. Cells were treated with 100ng/ml rhTRAIL as indicated, and subsequently fixed with 4% v/v PFA. Protein proximity was subsequently determined using the Duolink II Fluorescence assay according to the manufacturer's instructions (Olink Bioscience, Uppsala, Sweden). Briefly, after blocking non-specific binding sites with BSA, cells were incubated with anti-CRT (ab2907), anti-TRAIL (B-S23), anti-TRAILR2 (Hs201) or anti-CD47 (B6H12.2) for 1 hour at 22°C. Cells were subsequently washed and incubated with the Duolink plus and minus PLA-probes, followed by a ligation step to hybridize the probes. Next, the fluorescent signal was enhanced by rolling circle amplification, and nuclei were stained with DAPI mounting medium. Fluorescent signal was examined using an EVOS fl fluorescent microscopy (Advanced Microscopy Group, Bothell, USA).

Immunoprecipitation

For TRAIL immunoprecipitation, 15 x 10^6 cells were stimulated with 100 ng/mL of HA-TRAIL in 7.5 mL medium for the indicated times at either 4°C or 37°C. Cells were then washed three times with cold PBS and lysed in 1 ml lysis buffer (1% Triton, 20 mM of Tris-HCl (pH 7.5), 150 mM of NaCl, 1 mM of CaCl, 1 mM of MgCl and Protease inhibitor cocktail). Lysates were pre-cleared with Protein G-sepharose and immunoprecipitated overnight at 4°C with anti-HA sepharose beads. For CRT or TRAIL receptor immunoprecipitations, cells were stimulated with 100 ng/mL HA-TRAIL as described above, washed and lysed in Triton-containing lysis buffer. Lysates were pre-cleared as described and 10 µg of immunoprecipitating antibody (anti-CRT - Ab39818 or anti-TRAILR2 - HS-202) was added. In both cases, beads were then washed four times with lysis buffer and immunoprecipitates were eluted in SDS-sample buffer prior to being used in immunobloting.

Western blotting

Human melanoma cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation in PBS containing 0.05% Tween-20 and 5% w/v powdered milk. Immunoblots were subsequently incubated with specific primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody, and were developed by Super signal west dura ac-

cording to the manufacturer's protocol (Thermo Scientific).

Analysis of cell death

For cell death assays, melanoma cancer cells were treated for 16 hours with rhTRAIL (100ng/mL) in the presence or absence of soluble CRT as indicated, after which apoptosis was assessed in tumor cells by loss of mitochondrial membrane potential ($\Delta\Psi$) as previously described (22), using an Accuri C6 flow cytometer. For inhibition of apoptosis using sCRT cells were treated using rhTRAIL and sCRT essentially as described before for FasL (8).

Statistical analysis

Data reported are mean values±SD of three independent experiments. Data was analyzed by one-way ANOVA followed by Tukey-Kramer post test or, where appropriate, by two-sided unpaired Student's t-test. Where indicated * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

Results

Calreticulin directly binds to TRAIL and co-localizes with TRAILR2 on melanoma cells

To confirm the initial reports on the direct interaction of CRT with TRAIL, a catching type ELISA was performed with TRAIL on CRT coated plates. As anticipated, addition of TRAIL to CRT-coated ELISA plates resulted in the specific and dose-dependent interaction of TRAIL with CRT (Fig.1A). To confirm that this interaction also occurred on the cell surface of cells, A375M melanoma cells were treated for 3h with TRAIL. A375M cells were chosen because they express only TRAILR2 on their cell surface. Using fluorescent microscopy, no CRT signal was detected on the cell surface of untreated A375M cells (Fig. 1B). However, treatment with TRAIL resulted in the presence of a clear patched pattern of cell surface CRT (Fig. 1B). This CRT staining partly overlapped with cell surface expressed TRAILR2 (Fig. 1B). These results were confirmed using confocal microscopy, where again cell surface staining of CRT on untreated control cells was not detectable (data not shown). In contrast, in TRAIL-treated A375M cells, a clear punctuate cell surface expression of CRT was visible (Fig. 1C). The majority of this cell surface expressed CRT was co-localized with TRAILR2 (Fig. 1C). Reversely, only part of cell surface expressed TRAILR2 was co-localized with CRT (Fig. 1C). Importantly, PLA further confirmed that CRT and TRAIL (Fig. 2A), as well as CRT and TRAILR2 (Fig. 2B), were in close proximity (<40nm or ~4-12 proteins) after treatment with TRAIL. Furthermore, the PLA staining indicates that CRT forms complexes with both TRAIL and TRAILR2 on the cell surface of A375M cells. Interestingly, CRT dissociated from CD47, a known CRT binding partner (Fig. 2C). Together these data clearly establish the co-localization of CRT with TRAIL on the cell surface of TRAIL-treated, but not untreated, A375M cells.

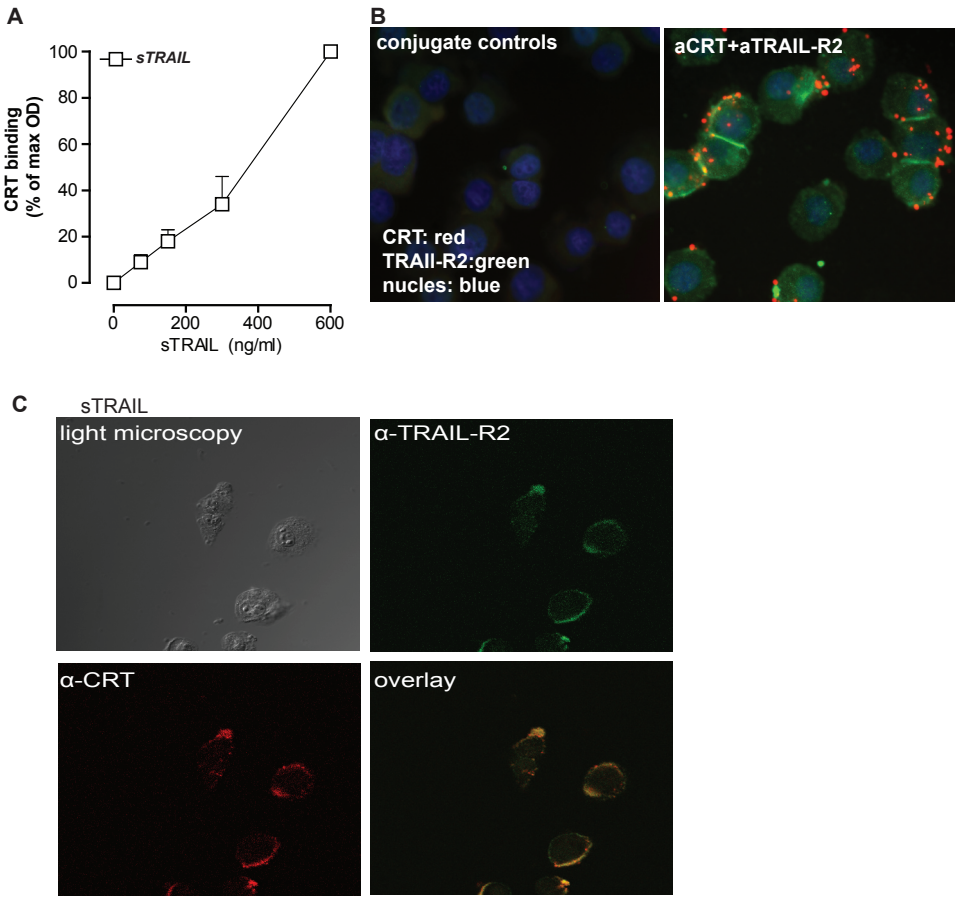

Figure 1. Calreticulin binds to TRAIL and associates with TRAILR2. A Calreticulin was coated and binding of increasing concentrations of TRAIL were assessed in a catch-type ELISA. **B** A375M melanoma cells were treated with 100 ng/mL rhTRAIL for 1h, double stained for CRT (red) and TRAILR2 (green) and analyzed using fluorescent microscopy. **C** A375M cells were treated with 100 ng/mL rhTRAIL for 1h, double stained for CRT (red) and TRAILR2 (green) and analyzed using confocal microscopy.

Calreticulin associates with the TRAILR2-DISC via TRAIL

Since CRT co-localized with TRAILR2 only after treatment of melanoma cells with TRAIL, we next evaluated whether TRAIL-treatment resulted in the formation of a DISC to which CRT might be recruited. Indeed, immunoprecipitation of HA-tagged TRAIL after 15 and 180 minutes of treatment with TRAIL at 37°C co-immunoprecipitated a complex that included CRT, FADD, and pro-caspase-8 (Fig.3A). Of note, caspase-8 was predominantly present

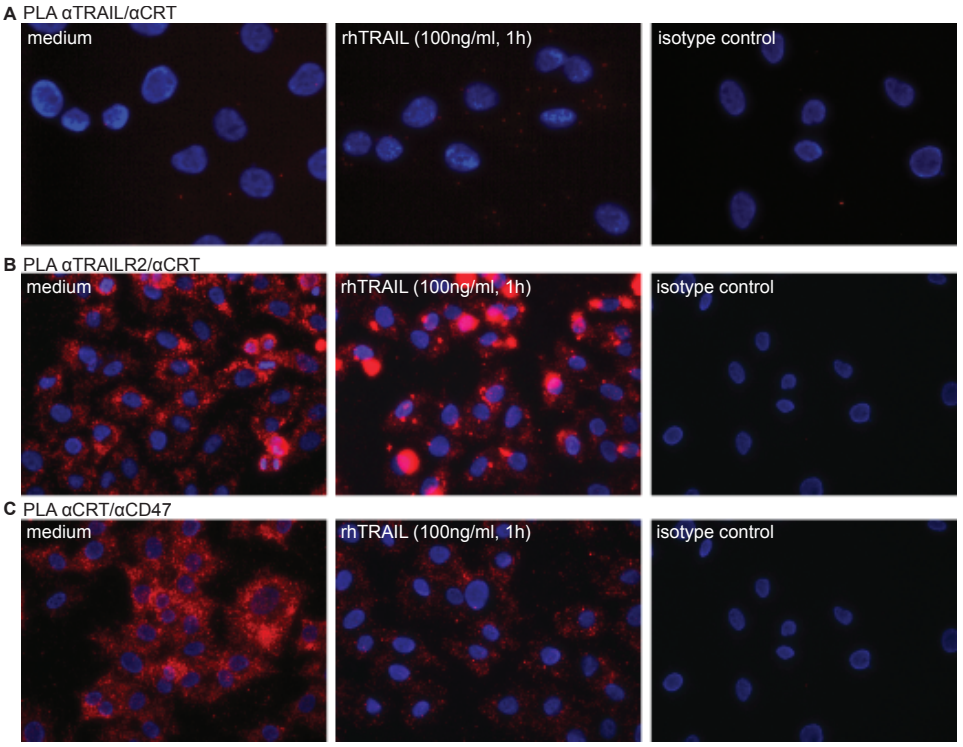

Figure 2. Calreticulin associates with TRAIL and TRAILR2. A375M melanoma cells were treated with 100 ng/mL rhTRAIL for 1h, incubated with mAbs directed against CRT and **A** TRAIL, **B** TRAILR2 or **C** CD47 and subsequently used for proximal ligation assay (PLA).

in its full-length pro-enzyme (p54) form after 15 min. of treatment, but was also found in its intermediate processed p41/43 form (Fig.3A). This p41/43 was further increased after 3h of treatment (Fig. 3A). Treatment on ice also resulted in the formation of the TRAIL-associated complex comprised of typical DISC components and CRT (Fig.3A). However, partial caspase-8 processing on ice was only detected after 3h of treatment with TRAIL (Fig.3A). To further confirm these data we performed a reciprocal CRT-immunoprecipitation. Four different anti-CRT antibodies were evaluated for their ability to co-immunoprecipitate TRAIL in TRAIL-treated A375M cells. Of these four antibodies, IP with Ab39818 resulted in the highest amount of TRAIL co-precipitated with CRT (data not shown). Thus, further IP experiments were performed with Ab39818. After 3h of treatment with recombinant TRAIL, immunoprecipitation with Ab39818 resulted in the co-precipitation of TRAIL, TRAILR2, as well as initiator caspase-8 (Fig.3B). Of note, 3h treatment with TRAIL at 0˚C co-precipitated mainly the full-length p54 form of caspase-8, whereas treatment with TRAIL for 180 min. at

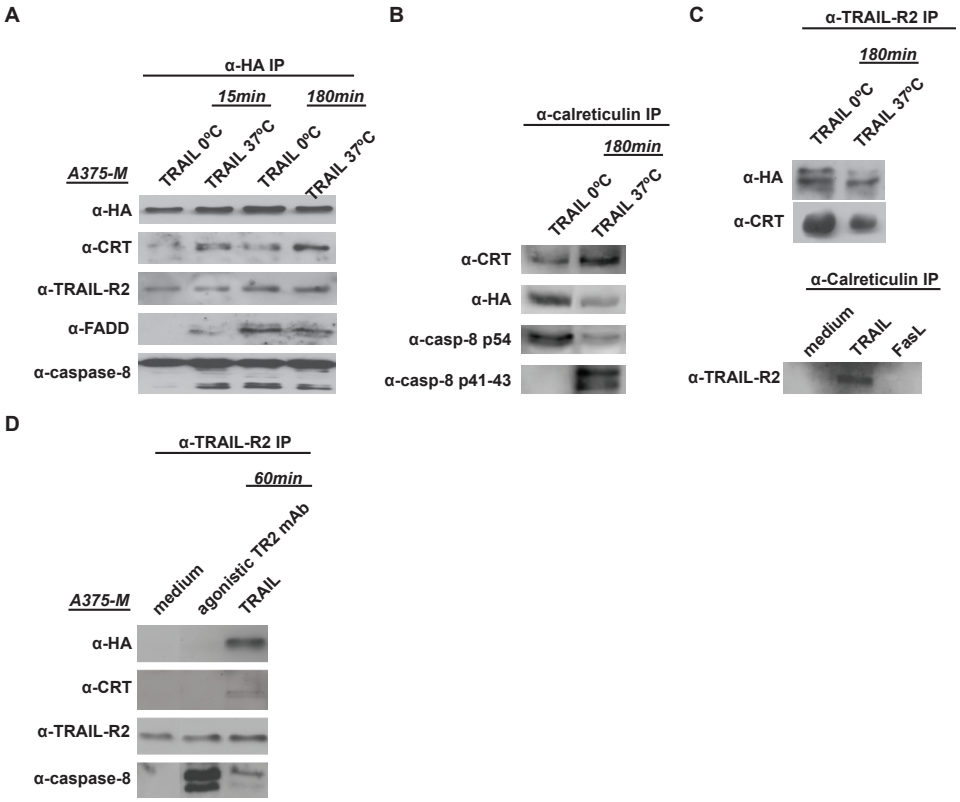

Figure 3. Calreticulin is recruited with TRAIL to the TRAILR DISC. A A375M cells were treated with HA-TRAIL for 15 or 180 min. at the indicated temperatures. Cells were subsequently lysed and HA-TRAIL was precipitated via affinity tag purification. Precipitates were subsequently probed for the presence of HA, CRT, TRAILR2, FADD and caspase-8. **B** A375M melanoma cells were treated with HA-TRAIL for 180 min. at the indicated temperatures. Cells were subsequently lysed and CRT was precipitated using anti-CRT Ab39818. Precipitates were subsequently probed for the presence of CRT, HA and caspase-8. **C** (Upper panel) A375M melanoma cells were treated with HA-TRAIL for 180 min. at the indicated temperatures. Cells were subsequently lysed and TRAILR2 was precipitated using anti-TRAILR2 mAb HS-202. Precipitates were subsequently probed for the presence of HA and CRT. (lower panel) A375M melanoma cells were treated with HA-TRAIL or FasL for 180 min. at 37°C. Cells were subsequently lysed and CRT was precipitated using anti-CRT Ab39818. Precipitates were subsequently probed for the presence of TRAILR2. **D** A375M melanoma cells were treated with HA-TRAIL or agonistic TRAIL mAb for 60 min. at 37°C. Cells were subsequently lysed and TRAILR2 was precipitated using the agonistic TRAIL mAb. Precipitates were subsequently probed for the presence of HA, CRT, TRAILR2 and caspase-8.

37˚C mainly precipitated the p41/43 form of caspase-8 (Fig. 3B).

Subsequently, TRAILR2 was precipitated to identify whether CRT and TRAIL associated with TRAILR2 in one, or in two separate complexes. As expected, precipitation of TRAILR2

resulted in the co-precipitation of TRAIL (Fig. 3C). In addition, TRAILR2 immunoprecipitation also resulted in the co-precipitation of CRT (Fig. 3C), which suggests that CRT associated with TRAIL and TRAILR2 in one complex. To exclude that CRT interacted with TRAILR2 in the absence of TRAIL, CRT was immunoprecipitated from untreated cells, TRAIL-treated cells and control FasL-treated cells. Importantly, TRAILR2 was only co-precipitated in TRAIL-treated cells (Fig. 3C). To further confirm that the interaction of CRT with TRAIL was necessary for its association with TRAILR2, A375M cells were treated with TRAIL or agonistic anti-TRAILR2 antibody. Subsequent IP of TRAILR2 only resulted in the co-immunoprecipitation of CRT after treatment with TRAIL and not with agonistic TRAILR2 antibody, whereas caspase-8 was detected in cells treated either with TRAIL or agonistic TRAILR2 antibody (Fig. 3D).

Calreticulin association with the TRAILR2-DISC is time-dependent

Since CRT associated with TRAIL in A375M cells, we next investigated whether this interaction was constant or transient. To this end, A375M cells were treated with TRAIL for various time-points, after which TRAIL was immunoprecipitated. In these time-course experiments, IP of TRAIL resulted in the co-precipitation of CRT. However, CRT association was minimal at early time-points, increased to a maximum association after ~1h and appeared to reach a stable level after 2 and 3 hours of treatment (Fig. 4A). IP of CRT in an analogous time-course confirmed a peak of interaction with TRAIL at 1h followed by a decrease to stable levels of CRT/TRAIL interaction after 2-3 h (Fig. 4B). Of note, association of CRT with the pro-form of caspase-8 rapidly reached a maximum at initial time-points of 5 and 15 minutes after which association started to steadily decrease in time (Fig. 4C). At the same time, the association of the processed form of caspase-8 increased in time until 1h of treatment and then declined to constant level at 2-3 hours of treatment (Fig. 4C). This maximal processing of caspase-8 was in time followed by the activation of effector caspase-3 as determined by fluorescent activity assay (Fig. 4D). These data were confirmed using PLA analysis that demonstrated a time-dependent increase in TRAILR2-CRT proximity with a maximum association after ~1h of treatment (Fig. 5).

Calreticulin association with the TRAILR-DISC is independent of execution of apoptosis

Next we determined whether the association of CRT with TRAIL was dependent on TRAIL-induced apoptosis. To this end, Ramos B-cells overexpressing the anti-apoptotic DISC component cFLIP (Ramos.cFLIP$_L$ and Ramos.cFLIP$_s$) were treated with TRAIL. Interestingly, IP of TRAIL resulted in the co-precipitation of CRT in Ramos cells, but also in Ramos.cFLIP$_L$ and Ramos.cFLIP$_s$, indicating that activation of caspases was not required for the association of CRT with TRAIL (Fig. 6A). Similarly, treatment of A375M cells with pan caspase-inhibitor zVAD-fmk did not abrogate TRAIL-CRT association (Fig. 6B). Further-

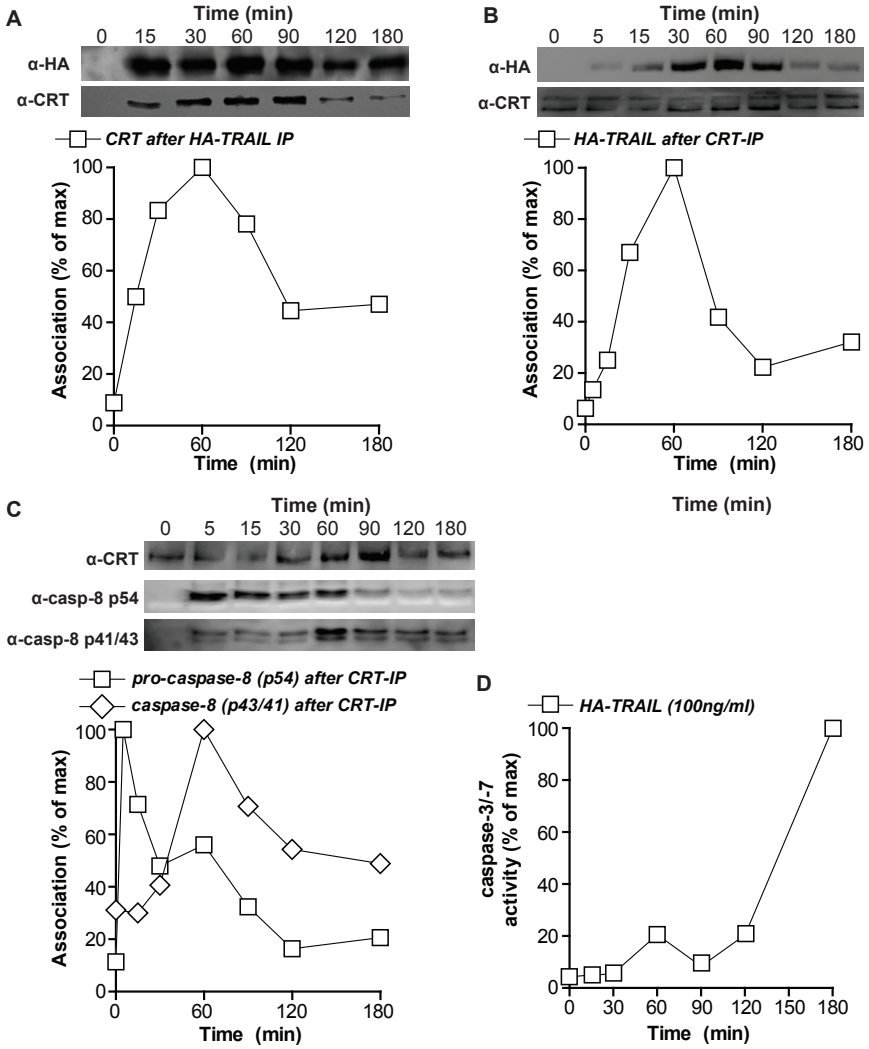

Figure 4. Calreticulin association with the TRAILR-DISC is time-dependent. A A375M cells were treated with HA-TRAIL at 37°C for the indicated time-points. Cells were subsequently lysed and HA-TRAIL was precipitated via affinity tag purification. Precipitates were subsequently probed for the presence of CRT. **B** A375M cells were treated with HA-TRAIL at 37°C for the indicated time-points. CRT was precipitated using anti-CRT Ab39818. Precipitates were subsequently probed for the presence of HA. **C** A375M cells were treated with HA-TRAIL at 37°C for the indicated time-points. CRT was precipitated using anti-CRT Ab39818. Precipitates were subsequently probed for the presence of caspase-8. **D** A375M melanoma cells were treated with HA-TRAIL at 37°C for the indicated time-points and caspase-3 activity was assessed.

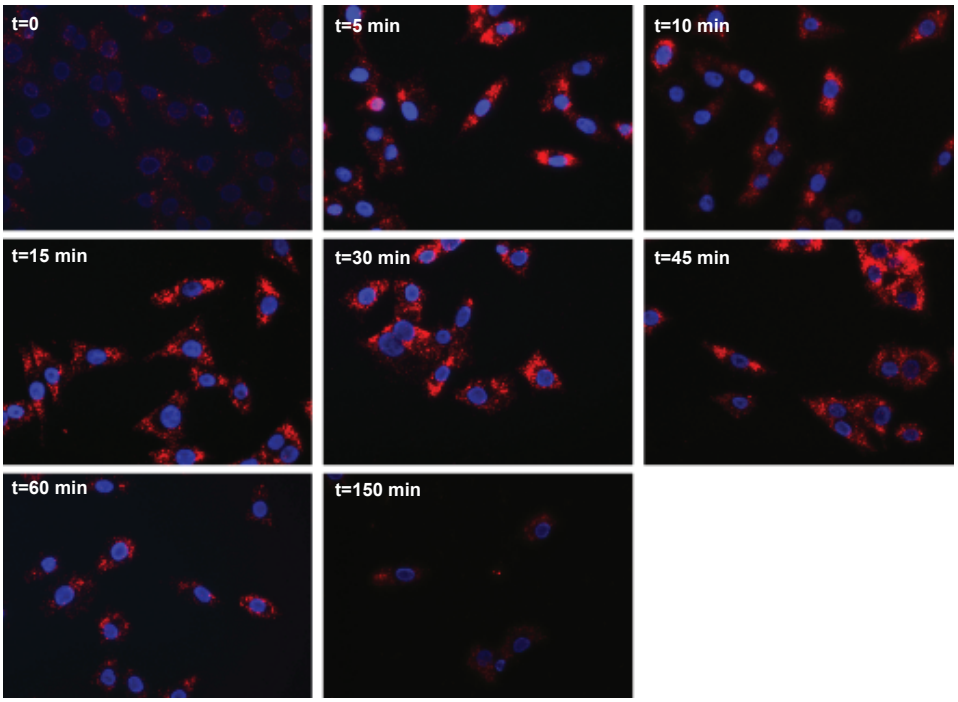

Figure 5. Calreticulin association with the TRAILR-DISC is time-dependent. A375M melanoma cells were treated with 100 ng/mL rhTRAIL at 37°C for the indicated time-points, incubated with mAbs directed against CRT and TRAILR2 and subsequently used for proximal ligation assay (PLA).

more, A375M cells overexpressing a dominant negative mutant of FADD (FADD-DED) that is unable to signal apoptosis still showed TRAIL/CRT co-localization after TRAIL treatment (data not shown).

Next, we assessed whether cytoskeletal reorganization might be required for the CRT/TRAIL association. In Ramos.Ezrin cells that overexpress a dominant negative form of Ezrin, whereby cytoskeletal reorganization is blocked, TRAIL was not co-precipitated with CRT (Fig. 6A).

Taken together, these data indicate that whereas CRT association is independent of the execution of TRAIL-mediated apoptosis, the reorganization of the cytoskeleton is required for CRT/TRAIL association.

Calreticulin does not influence the apoptotic activity of TRAIL

Since the association of TRAIL with CRT occurred independently of caspase activation, we speculated that CRT might modulate TRAIL induced apoptosis at the cell surface. As expected, treatment of Ramos, Ramos.cFLIP$_L$, Ramos.cFLIP$_s$ and Ramos.Ezrin with TRAIL

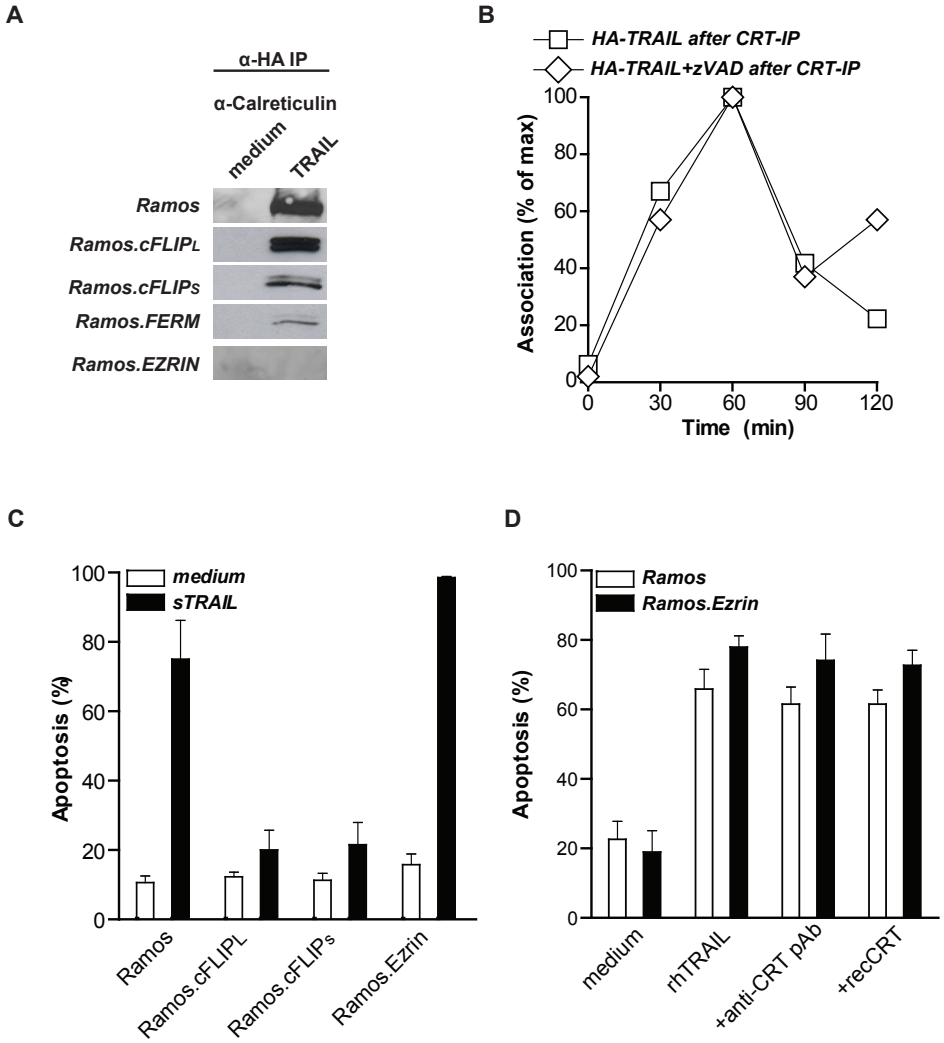

Figure 6. Calreticulin association with the TRAILR-DISC is time-dependent. A Ramos, Ramos.cFLIPL, Ramos.cFLIPS, Ramos.FERM and Ramos.Ezrin cells were treated with HA-TRAIL for 1h at 37°C. Cells were subsequently lysed and HA-TRAIL was precipitated via affinity tag purification. Precipitates were subsequently probed for the presence of CRT. **B** A375M cells were treated with HA-TRAIL in the presence or abscence of zVAD-fmk. Cells were lysed at the time points indicated and CRT was precipitated. Precipitates were subsequently proped for HA-TRAIL. **C** Ramos, Ramos.cFLIPL, Ramos.cFLIPS and Ramos.Ezrin cells were treated with rhTRAIL for 16h and apoptosis was assessed. **D** Ramos and Ramos.Ezrin cells were treated with rhTRAIL in the presence or absence of CRT or CRT-neutralizing antibody for 16h and apoptosis was assessed.

triggered apoptosis in Ramos, but not in Ramos.cFLIP$_L$ and Ramos.cFLIP$_s$ (Fig.6C). In contrast, Ramos.Ezrin cells, where CRT/TRAIL association does not occur, were slightly more sensitive to TRAIL-induced apoptosis then wild-type Ramos cells. However, treatment with exogenous CRT or an anti-CRT polyclonal antibody that is known to neutralize CRT activity in phagocytosis assays had no effect on apoptosis induced by TRAIL in either Ramos or Ramos.Ezrin (Fig. 6D), indicating that the increased sensitivity of Ramos.Ezrin cells to TRAIL is likely to be CRT-independent or alternatively cannot be influenced by extra-cellular CRT.

Discussion

In many cell types, but particularly in tumor cells, the trafficking of CRT from the ER to the cell surface in association with other ER proteins (23) and interaction with apoptotic regulation proteins has been described (6). A previous study has reported the interaction of CRT with TRAIL in vitro (9). Here we report on the cell-surface interaction of the pro-phagocytic molecule CRT with the pro-apoptotic TNF-family member TRAIL. In addition, our data establish that calreticulin transiently associated with the TRAILR Death DISC in A375M melanoma and leukemic Ramos B-cells.

Treatment of cells with TRAIL induced a rapid (<5min.) recruitment of CRT towards the TRAILR DISC independent of proximal caspase signaling, suggesting this was a pre-apoptotic event. Furthermore, recruitment of CRT to the TRAILR DISC was contingent on the presence of TRAIL and did not occur upon treatment with FasL or an agonistic TRAILR2 mAb. Together, these data indicate that the physical interaction of TRAIL and CRT mediates recruitment of CRT to TRAILR2. However, our data do not exclude simultaneous binding of CRT to both TRAIL and TRAILR2. This translocation of CRT to TRAILR2 was dependent on cytoskeletal rearrangements since overexpression of a double negative Ezrin mutant (DN-Ezrin), incapable of binding to actin, abolished the interaction. Interestingly, these Ramos.Ezrin cells were more sensitive to TRAIL-mediated apoptosis, with ~20% more cell death in Ramos.Ezrin compared to Ramos. However, addition of exogenous CRT had no effect on this differential induction of apoptosis by TRAIL, indicating that these effects were likely CRT-independent. In line with this, a recent report indicates that Ezrin functions as a negative regulator of both FasL and TRAIL mediated apoptosis in T-cells and functional inhibition of Ezrin, either by siRNA or expression of DN-ezrin, accelerates induction of apoptosis by TRAIL (24).

Our immunohistochemical data indicate changes in the cell surface distribution of CRT on TRAIL-treated cells. Such changes in CRT-localization on apoptotic cells have previously been reported for UV-irradiated cells and appear to reflect changes towards a more pro-phagocytic phenotype (25). Indeed, CRT is released from anti-phagocytic binding partner CD47 on cells undergoing UV-induced apoptosis and re-localizes in membrane patches

rich in phosphatidyl serine (PS), another pro-phagocytic signaling molecule. Not surprisingly, this reorganization enhances uptake of apoptotic cells by phagocytes (25). In this current study, the proximal ligation studies also identified a reduced co-localization of CRT and CD47 in A375M cells undergoing TRAIL-induced apoptosis. These data support a model of CRT release from CD47 on cells undergoing apoptosis and indicate that treatment with TRAIL might function similar to UV-radiation in releasing CRT from an anti-phagocytic complex.

Although involved in enhancing phagocytosis, CRT is also a key determinant of whether cell death provokes an immune response. Chemotherapeutic agents that simultaneously induce apoptosis and elicit the translocation of CRT from the ER to the cell surface are more immunogenic in mouse models when compared to agents that do not induce CRT exposure (4). Moreover, primary patient-derived human cancer cells treated with CRT-exposing agents (anthracylins) display enhanced uptake by immature DCs and subsequent DC maturation (26). Interestingly, TRAIL was also recently found to induce this CRT exposure in a caspase-independent manner (27). These findings are similar to the caspase-independent recruitment of CRT to the TRAILR2 complex reported here by us. As such, it is tempting to speculate that while TRAIL might initially induce immunogenic CRT exposure or re-localization, the association with the TRAIL might render CRT unable to affect an immune response.

In conclusion, we provide evidence that calreticulin associates in a caspase-independent manner with the pro-apoptotic TRAILR2 DISC in melanoma and leukemia cells, but does not affect the induction of apoptosis by TRAIL. Therefore the role of calreticulin differs from the FasL and L,D-MDP-induced apoptosis, which appears to be regulated to some extent by association with CRT. Further studies are required to fully elucidate the role of CRT with TRAIL and TRAILR2 DISC.

Acknowledgements

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Summary and perspectives

The research reported on in this thesis is dedicated to the preclinical development of immunotherapeutic strategies against cancer using the tumoricidal protein TRAIL. TRAIL is considered to be a promising therapeutic agent due to its prominent apoptotic activity towards many different types of cancer and its apparent lack of toxicity towards normal human cells. As such, a recombinant form of soluble TRAIL (sTRAIL) is currently undergoing early phase clinical evaluation in cancer patients. However, (pre-) clinical research has identified several features of the TRAIL/TRAIL-receptor biology that might limit the efficacy of recombinant sTRAIL in patients. These include the ubiquitous expression of TRAIL-receptors (TRAILR) throughout the human body, and the short serum half life (~ 30 min.) of TRAIL. In addition, recombinant sTRAIL is less efficient at inducing apoptosis in cancer cells than the full-length membrane-bound form of TRAIL.

To overcome these potential drawbacks, we have pursued a TRAIL-based therapeutic approach in which recombinant soluble TRAIL is genetically fused to a cancer cell surface targeting domain. Typically, the targeting domain is a so-called antibody fragment (scFv), directed against a pre-defined tumour-associated cell surface antigen. As detailed in **chapter 2**, this targeting domain ensures selective delivery of high levels of sTRAIL at the cell surface of tumour cells. Once bound via the targeting domain, sTRAIL is converted to a membrane-bound form of TRAIL and regains its full tumoricidal activity. In earlier studies, the feasibility and efficacy of this immunotherapeutic strategy has been evaluated for various types of cancer, including solid cancers and haematological malignancies.

In this thesis, the concept of tumour-targeted delivery of sTRAIL was further developed in preclinical studies with an emphasis on eliminating metastasis and disseminated disease (**chapters 3** to **5**). In addition, this tumour cell-targeted approach was adapted to an immune cell-targeted approach with the goal of augmenting anti-tumour activity of T-cells. As demonstrated in **chapter 6**, selective delivery of high levels of TRAIL to the cell surface of T-cells, a key tumoricidal immune effector cell population, strongly augments their anti-tumour activity. This novel approach proved to be highly effective towards a host of different tumour cell types, including ovarian cancer and melanoma. The respective research chapters of this thesis are discussed in more detail below and perspectives for further (pre) clinical development are provided.

Epidermal Growth Factor Receptor (EGFR)-targeted TRAIL-based immunotherapy

In **chapters 3** and **4**, the therapeutic potential of delivering sTRAIL to the Epidermal Growth Factor Receptor (EGFR) was investigated. EGFR is an oncogenic growth factor that is over expressed on many carcinomas and is a well-established target for immunotherapy [1]. Inhibition of EGFR-signalling using antagonistic antibodies or small molecule inhibitors has already shown promising clinical efficacy [1,2]. Previously, we incorporated both of these elements into one recombinant therapeutic protein by generating an EGFR-targeted TRAIL

fusion protein comprised of an antagonistic anti-EGFR antibody fragment (scFv425) and sTRAIL. After binding to cancer cell-expressed EGFR, the anti-EGFR antibody fragment inhibited mitogenic signalling by EGFR, thereby sensitizing cells to TRAIL-induced apoptosis. At the same time, cell surface delivery of sTRAIL converts sTRAIL to membrane-bound TRAIL, yielding potent activation of TRAILR apoptotic signalling [3]. As described in **chapter 3**, scFv425:sTRAIL successfully eradicated disseminated EGFR-positive tumours in mice, yielding *in vivo* proof of concept for the tumoricidal activity of scFv425:sTRAIL. For this study, we used a gene therapeutic strategy (based on a replication-deficient adenovirus) to ensure the continuous *in vivo* production of scFv425:sTRAIL over extended periods of time (>30 days). At high serum levels (>10 µg/mL), scFv425:sTRAIL successfully eradicated established tumours. Importantly, scFv425:sTRAIL also significantly halted tumour growth at scFv425:sTRAIL serum levels as low as 60 ng/ml. We conclude that EGFR-targeted pro-apoptotic therapy using scFv425:sTRAIL may hold considerable promise for the treatment of malignant disease with intraperitoneal dissemination, such as ovarian cancer.

In chapter 3, the natural tropism of adenoviral particles for the liver was utilized to ensure continues production of circulating scFv425:sTRAIL in mice. However, for gene therapeutic purposes, several other modifications may be applied to enhance both anti-tumour activity as well as anti-tumour selectivity.

Adenoviral particles consist of a capsid core with extending fibers ending in so-called knob domains. Therefore, strategies to selectively retarget adenoviral particles to tumour cells generally focus on the modification of one of these structures (reviewed in [4]). First, adenoviral particles might be retargeted to tumour cells using bi-specific ligands that bind to the viral fiber knob domain and simultaneously to a tumour-associated antigen. Furthermore, adenoviral particles can be retargeted by incorporating targeting ligands into the adenoviral capsid proteins. In addition, fiber/knob chimeras have been generated in which the adenovirus knob is replaced by a knob from another virus, or alternatively, in which the entire adenovirus fiber is replaced with an artificial fiber. Adenoviral particles have also been modified by using so-called RGD-fibers. These RGD-fiber modified adenoviral particles display an enhanced infection activity of e.g. ovarian carcinoma cells [5].

Adenoviruses have also been engineered that are conditionally replicative (CRAd) (reviewed in [6]). These CRAd are designed to specifically infect cancer cells, replicate selectively, and then to spread to other cancer cells, without damaging untransformed cells. This has been achieved by deleting part of the viral genome to prevent replication in normal cells, but allow replication in tumour cells with genetic defects that complement the deleted viral genome functions. For instance, a 24 base pair deletion in the adenoviral gene E1A (dl922–947) results in an E1A viral protein that is unable to bind and inactivate the retinoblastoma tumour suppressor/cell cycle regulator protein preventing efficient viral replication in cells with an intact G1/S phase checkpoint.

In addition, viral replication and/or expression of the tumoricidal payload can be placed under the control of a tumour-specific or tumour-associated promoter (TSP), thereby restricting viral replication and toxicity to tumour cells. In one example, viral particles were generated in which a suicide gene was placed under the control of the promoter for EpCAM. EpCAM is a cell-surface molecule and is frequently over expressed on a host of carcinomas, including ovarian cancer. These adenoviral particles demonstrated a significantly reduced expression of the suicide gene in the liver and possessed enhanced tumoricidal activity [7].

Chapter 3 highlights a considerable promise of scFv425:sTRAIL for immunotherapy of cancer. However, the efficacy of the soluble TRAIL domain may be further optimized by using rationally designed sTRAIL variants. TRAIL has an intricate receptor system, comprising 5 different receptors, of which only TRAILR1 and TRAILR2 can transmit the apoptotic signal. The other three receptors, TRAILR3, TRAILR4 and osteoprotegerin (OPG), are so-called decoy receptors that are unable to convey apoptotic signalling. Interestingly, the expression of the various TRAIL receptors per se does not have a reliable predictive value with regard to sensitivity to TRAIL-induced cell death.
To enhance the selectivity of sTRAIL for one or both agonistic TRAIL-receptors and to reduce binding affinity for the antagonistic receptors, various research laboratories have generated variants of sTRAIL with one or more amino acid substitutions. In **chapter 4**, we evaluated the therapeutic potential of EGFR-targeted delivery of one of the reported variants of sTRAIL, sTRAILmR1-5, that was shown to have increased affinity for TRAILR1 and reduced affinity for TRAIL decoy receptors [8]. Indeed, scFv425:sTRAILmR1-5 had superior anti-cancer activity compared to the wild type scFv425:sTRAIL in ~50% of cell lines tested. Interestingly, although this TRAIL mutant was reported to only signal via TRAILR1, our data clearly indicate that upon EGFR-targeted delivery TRAILmR1-5 also efficiently triggers TRAILR2 apoptotic signalling. We conclude that fusion protein, anti-EGFR:TRAILmR1-5 has superior anti-tumour activity and may be an excellent candidate for further (pre-) clinical development.
Similarly, several lines of evidence suggest that incorporating a TRAILR2-selective sTRAIL mutant into our scFv:sTRAIL fusion protein format may yield novel therapeutics with unique anti-tumour activity. First, TRAILR2 is often over expressed compared to TRAILR1 in tumour cells. Second and as also described above, TRAILR2 signalling is only efficiently triggered in the context of membrane-bound TRAIL whereas TRAILR1 signalling can also be efficiently triggered by sTRAIL. As such, a scFv:TRAILR2-selective mutant fusion protein would only gain full fledged TRAILR2-mediated apoptotic activity once bound to the target TRAILR2-positive cell, yet remain inactive towards TRAILR1 while “en route”. This untargeting of TRAILR1 could potentially prevent ubiquitous activation of TRAILR1 and might

proof to be beneficial in terms of unwanted toxicity. Of note, studies on cynomolgus monkey hepatocytes indicate that a TRAILR2 selective mutant only induces hepatocyte toxicity after antibody-mediated crosslinking [9]. Therefore, selective delivery of a scFv:TRAILR2-selective mutant to a tumour cell surface antigen not expressed on hepatocytes could further preclude unwanted toxicity towards untransformed cells.
Obviously, rational combinatorial delivery of TRAILR1 and TRAILR2 selective mutants to different tumour cell surface antigens may enhance tumour-selective induction of apoptosis even further.

Bi-functional scFv:sTRAIL fusion proteins combining anti-metastatic and pro-apoptotic activity

It is well-established that the efficacy of protein-based therapeutics may be hampered by the inherent limited diffusion rate of macromolecules over multiple cell layers. In the case of solid cancers, tumour penetration is further limited by high intra-tumoural pressure. Therefore, the most straight-forward anti-cancer application of TRAIL-based immunotherapy as described in this thesis is after (or possibly during) cytoreductive intervention, such as surgical debulking and/or chemo/radiotherapy. After cytoreductive intervention, a particularly important and as of yet unsolved problem is the potential presence of circulating cancer cells that, after period of minimal residual disease (MRD), ultimately trigger formation of local and/or distant metastasis. Prevention and/or inhibition of metastases formation by circulating or occult cancer cells is therefore paramount to achieving curative treatment. To evaluate the utility of our immunotherapeutic approach towards such circulating/metastatic cancer cells, we constructed and evaluated the melanoma-targeted TRAIL fusion protein anti-MCSP:TRAIL (**chapter 5**). Melanoma was chosen in this study as it is a prime example of metastatic disease. This is exemplified by the fact that surgical resection of localized primary melanomas often yields curative treatment, yet metastatic melanoma has a very dismal prognosis (survival rate of <9 months) and is essentially incurable. The melanoma chondroitin sulphate proteoglycan (MCSP) was chosen as a suitable target antigen, not only based on its high expression on metastatic melanoma cells, but also since MCSP is thought to contribute to the metastatic potential of melanoma cells. Therefore, fusion protein anti-MCSP:TRAIL was designed to inhibit MCSP-mediated pro-metastatic signalling, while simultaneously triggering TRAIL-mediated apoptotic cell death. Indeed, anti-MCSP:TRAIL demonstrated strong apoptotic activity towards a panel of melanoma cell lines *in vitro*. Importantly, treatment with anti-MCSP:TRAIL strongly inhibited growth of human melanoma xenografts in mice. Intriguingly, *in vitro* analysis indicated that anti-MCSP:TRAIL was over 100-fold more effective in eliminating melanoma cells in metastatic settings (3D colony forming cultures) than in adherent 2D cultures. This potent activity against colony forming cells was found to be the result of combined inhibition of tumorigenic MCSP-signalling and

induction of apoptosis by TRAIL. Therefore, anti-MCSP:TRAIL holds considerable promise for the treatment of MRD and elimination of metastatic melanoma cells.
Of note, tumorigenic signalling has also recently been reported for the target antigen EpCAM [10]. Upon cell-cell contact between neighbouring cells, the intracellular region of EpCAM (EpCAM IntraCellular Domain; EPICD), was found to be cleaved from the membrane upon which it translocated to a transcription factor complex in the nucleus. In the nucleus, EPICD activated the proto-oncogene c-Myc resulting in pro-mitogenic signalling. In light of this, we speculated that the potent anti-tumour activity we have previously found for the EpCAM-specific fusion protein scFvC54:sTRAIL may have been partly related to inhibition of EpCAM processing and concomitant inhibition of mitogenic cell signalling pathways. Therefore, we recently examined whether treatment of HEK293 cells stably expressing EpCAM-YFP (where the YFP tag is located on the EPICD domain) with scFvC54:sTRAIL could abrogate EpCAM cleavage and translocation of EPICD. Interestingly, treatment of these cells with scFvC54:sTRAIL blocked EpCAM cleavage induced by homotypic interaction with soluble EpCAM and prevented intracellular translocation of EPICD (Fig. 1). Currently, further studies into these anti-tumour effects of scFvC54:sTRAIL in the context of MRD are ongoing.

Enhancing the anti-tumour activity of immune effector cells by targeted delivery of TRAIL

TRAIL is an integral part of the immune system and in this capacity serves to prevent and control the development and progression of malignant cells. Most notably, transmembrane TRAIL appears to be the main immune effector mechanism by which resident natural killer (NK) cells prevent circulating tumour cells to invade the liver [11]. Thus, a clear rationale exists for ensuring that immune effector cells have high levels of tumoricidal TRAIL on their surface. In **chapter 6**, TRAIL was selectively delivered to the cell surface of T-cells and antitumor activity of such TRAIL-“armed” T-cells was evaluated. T-cells were chosen for several reasons. First of all, there is a window of opportunity for displaying TRAIL on T-cells, since these cells normally do not or only minimally express TRAIL on the cell surface. Second, large infiltrates of T-cells can be detected in many tumours and the presence of such tumour infiltrated T-cells (TILs) is often associated with a better prognosis for the patient [12]. Apparently, TILs are able to (temporarily) control tumour growth. Finally, T-cells already exert a strong intrinsic anti-tumour activity that relies on immune effector molecules other then TRAIL.
As described in chapter 6, TRAIL was selectively delivered to the cell surface of T-cells using an anti-CD3 antibody fragment (anti-CD3) or the CD7 ligand K12. The resultant fusion proteins, anti-CD3:TRAIL and K12:TRAIL, proved to significantly enhance the anti-cancer activity of T-cells *in vitro* and *in vivo*. As anticipated, K12:TRAIL enhanced the tumoricidal

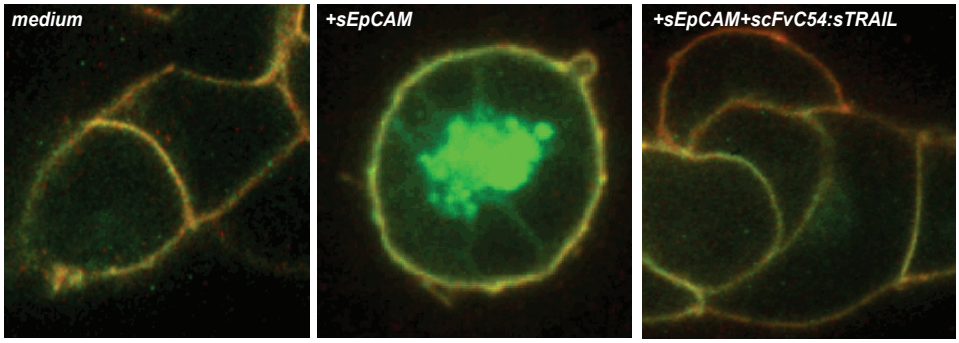

Figure 1. ScFvC54:sTRAIL inhibits sEpCAM-mediated cleavage of EpCAM and intrecellular translocation of EpICD. HEK293.EpCAM-YFP cells were treated with sEpCAM to induce EpICD cleavage in the presence or abscence of scFvC54:sTRAIL. Cells were subsequently counter-stained for membrane-localized EpCAM using anti-EpCAM antibody Moc31-PE and EpICD-YFP translocation was visualized using confocal microscopy.

activity of T-cells by activation of pro-apoptotic TRAIL-receptor signalling in tumour cells. Surprisingly, the potentiating activity of anti-CD3:TRAIL was found to be predominantly due to activation of intrinsic T-cell mediated cytotoxicity (e.g. granzyme/perforin signalling). CD3 is a major signalling component of the T-cell receptor (TCR) complex and, as such, can induce T-cell activation after binding of antibodies or antibody fragments. Intriguingly, our data indicate that binding of the anti-CD3 domain of anti-CD3:TRAIL induces T-cell activation only after crosslinking via TRAILR2 on the tumour cell surface. Thus, depending on the target antigen of choice, a TRAIL fusion protein can have a strikingly different activity profile.

The anti-tumour activity of anti-CD3:TRAIL is reminiscent of so-called T-cell redirecting bi-specific antibodies. These antibodies bind to an antigen on the T-cell (for instance CD3) and an antigen on the tumour cell (for instance EpCAM). As a result of CD3 signalling, T-cells become activated. Subsequently, binding of the bi-specific antibody to EpCAM induces proximity-induced release of granzymes/perforins and subsequent elimination of EpCAM-positive tumour cells (Figure 2). Of note, combination of a bi-specific anti-CD3x-anti-EpCAM antibody with K12:TRAIL synergistically enhanced the anti-tumour activity of T-cells (unpublished data).

The concept of bi-specific antibodies has also been extended to so-called tri-bodies. Here, three distinct single-chain antibody fragments (scFvs) are genetically linked to enhance the avidity of the T-cell to the tumour cells. For instance, a tri-body directed against CD3, EpCAM and EGFR simultaneously binds T-cells via CD3 and tumour cells via EpCAM and/or EGFR. Therefore, such a tri-specific antibody can redirect T-cells to tumour cells expressing both EpCAM and EGFR, but also to tumour cells expressing either EpCAM or EGFR

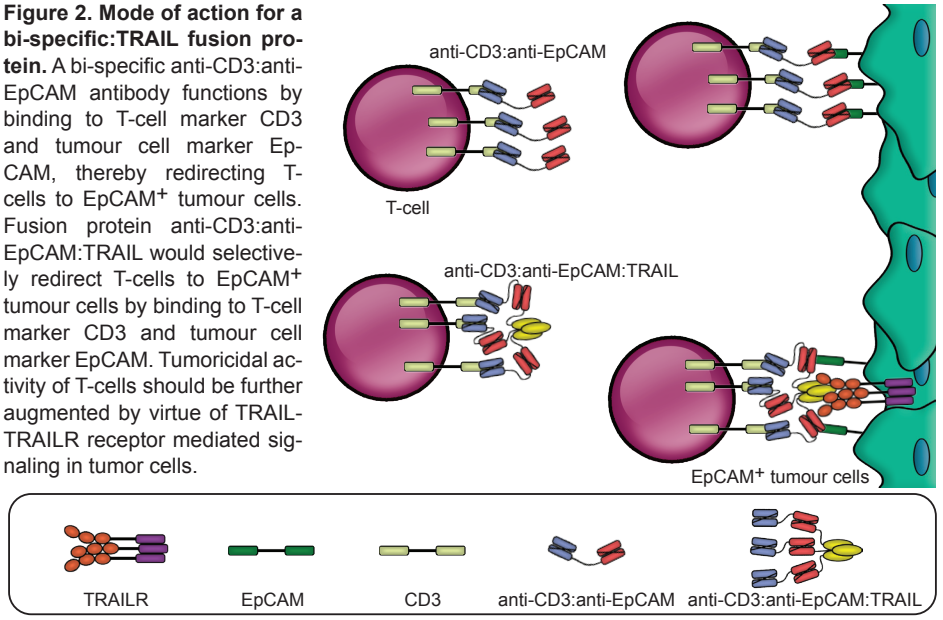

Figure 2. Mode of action for a bi-specific:TRAIL fusion protein. A bi-specific anti-CD3:anti-EpCAM antibody functions by binding to T-cell marker CD3 and tumour cell marker EpCAM, thereby redirecting T-cells to EpCAM$^+$ tumour cells. Fusion protein anti-CD3:anti-EpCAM:TRAIL would selectively redirect T-cells to EpCAM$^+$ tumour cells by binding to T-cell marker CD3 and tumour cell marker EpCAM. Tumoricidal activity of T-cells should be further augmented by virtue of TRAIL-TRAILR receptor mediated signaling in tumor cells.

alone. However, T-cells will preferentially bind tumour cells expressing both antigens simultaneously. As a result, off-target toxicity to untransformed cells expressing only EGFR or EpCAM is reduced. Based on the synergy between K12:TRAIL and bi-specific antibodies, TRAIL might be incorporated into such a tri-body approach. The resultant fusion protein, for instance anti-EpCAM:anti-CD3:TRAIL would redirect T-cells to the tumour cells while simultaneously “arming” T-cells with TRAIL (see Fig. 2).

However, the tumour micro-environment often contains immune-inhibitory molecules that appear to induce an anergic state in T-cells, preventing them from eliminating the cancer cells (reviewed in [13]). Indeed, both tumour-derived and tumour-induced molecules have been reported to induce anergy in TILs, which may serve to hamper the efficacy of K12:TRAIL/anti-CD3:TRAIL such as reported in chapter 6. Therefore, incorporation of antibody-based approaches that block these inhibitory factors into our strategy is warranted. Of particular interest in this respect is the use of a blocking anti-CTLA-4 antibody fragment. CTLA-4 is a T-cell inhibitory molecule triggered by members of the B-7 family, B7.1 and B7.2 (CD80 and CD86, respectively). Anti-CTLA-4 antibodies prevent binding of B7.1 and B7.2 to CTLA-4 and enhance T-cell mediated immune responses [14]. One such anti-CTLA-4 antibody, Ipilimumab was recently approved by the FDA for the treatment of advanced melanoma. In clinical trials, ipilimumab restored anti-melanoma T-cell activity, resulting in an increase in survival of ~2 year for most melanoma patients. Moreover, a small

subgroup of patients developed stable disease [14]. The use of an anti-CTLA-4 antibody fragment in the TRAIL fusion protein concept would exploit the dual-signalling concept by simultaneously inhibiting inhibitory T-cell signalling and enhancing the anti-tumour activity of T-cells. In this manner, a T-cell inhibitory signal would in essence be converted into a pro-apoptotic anti-tumour "weapon".
In addition to blocking inhibitory signals, antibody fragment that activate co-stimulatory signals on T-cells can also be incorporated into the TRAIL-based therapeutic strategy. Likely targets to this effect include 4-1BB, CD28 and inducible co-stimulator (ICOS). Indeed, antibodies that target these molecules have been shown to augment anti-tumour activity of T-cells in several preclinical studies [15-17].

An alternative way of enhancing this immunotherapeutic strategy is the use of the above highlighted TRAILR-selective TRAIL variants. For instance, T-cell activation and anti-tumour effects of anti-CD3:TRAIL depended on TRAILR2 binding/cross-linking. Therefore, it is tempting to speculate that the anti-tumour activity of the anti-CD3:TRAIL will be enhanced by genetically fusing a TRAILR2-selective mutant to anti-CD3 (anti-CD3:TRAILmR2). This fusion protein could induce maximal activation and tumoricidal potential of T-cells via cross-linking of CD3.

Several immunotherapeutic protocols have been developed that aim to redirect T-cells to engage and eliminate tumour cells. Mostly, these protocols rely on the viral transduction of T-cells with engineered T-cell receptors (eTCRs) (reviewed in [18]). These eTCRs enable T-cells to specifically recognize and activate upon encounter with tumour-associated cell surface molecules such as EpCAM. However, tumoricidal activity of such redirected T-cells may still be hindered by cancer cell resistant to T-cell effector mechanisms and/or tumour microenvironment-induced anergy of T-cells. Here, incorporation of TRAIL into viral transduction strategies might provide additional therapeutic benefits. This could be achieved by the transduction of T-cells with transmembrane TRAIL, or by transducing T-cells to produce a TRAIL fusion protein. The latter strategy is reminiscent of that presented in chapter 3 on the use of Ad-scFv425:sTRAIL. While adenoviruses cannot normally infect T-cells, bi-specific antibodies or viral fiber/knob modifications thereof might be used to redirect adenoviral particles towards T-cells [19]. Alternatively, viral vectors with an innate T-cell tropism such as lentiviruses could be exploited. In this respect, lentiviral particles have already been extensively used to deliver modified eTCR into T-cells (reviewed in [20]). A recent paper reported on the induction of immune response and ongoing remission 10 months after adoptive transfer of lentivirally transduced designer T-cells containing a chimeric eTCR [21]. Inclusion of lentiviral TRAIL fusion protein vectors might therefore be suitable for integration into these strategies.

The delivery of TRAIL to the cell surface of T-cells (chapter 6) can be readily incorporated into existing cancer immunotherapies and may therefore move rapidly into clinical evaluation. Pre-clinical side-by-side comparison of the above-described strategies will help identify the optimal form of targeted TRAIL for future clinical evaluation.

TRAIL in tumour immunogenicity

TNF-family members such as TRAIL are integral parts of the immune system and trigger cell death in target cells. Upon induction of apoptotic cell death, cancer cells are rapidly removed by phagocytosis. Typically, phagocytosis occurs in a non-immunogenic fashion, leading to the quiet removal of superfluous cells. However, recent studies highlight that anti-cancer therapies can also trigger immunogenic phagocytosis, which to a large degree depends on the pro-phagocytic receptor calreticulin (CRT).

CRT is an ER-resident protein involved in Ca^{2+} signalling and protein folding. However, treatment with certain apoptotic stimuli, including TRAIL, triggers translocation of CRT to the tumour cell surface. Here, CRT serves as an 'eat me' signal for phagocytes. Although normally non-immunogenic, the phagocytosis of pre-apoptotic cells with cell surface exposed CRT may lead to the induction of an anti-tumour immune response. Furthermore, CRT needs to dissociate from its binding partner CD47 on apoptotic cells in order for them to be effectively phagocytosed.

Based on these studies, and the recent finding that CRT can directly interact with TRAIL in an ELISA-type assay, we evaluated whether CRT may be involved in TRAIL/TRAIL-receptor mediated apoptotic cell removal. The data in **chapter 7** indicate that treatment of tumour cells with TRAIL induced the rapid pre-apoptotic association of CRT with TRAIL and TRAILR2. This association occurred during TRAIL-mediated DISC formation and activation of caspase-8, but preceded activation of caspase-3. Furthermore, CRT associated with the TRAILR DISC only in the presence of sTRAIL, but not when cells were treated with an agonistic TRAILR antibody. Therefore, association of CRT with the TRAILR DISC is likely mediated via sTRAIL. Concurrently to the association of CRT with TRAILR, CRT dissociated from CD47. Of note, dissociation of CD47 from CRT has been reported for cells undergoing apoptosis after exposure to UV-C radiation, and resulted in an enhanced clearance of apoptotic cells by phagocytes. Together, this indicates that TRAIL-induced apoptosis in tumour cells may promote tumour immunogenicity. However, TRAIL is generally considered to be a non-immunogenic inducer of apoptosis. Therefore, the pre-apoptotic association of CRT with TRAIL might also serve to prevent an immune response. Although further studies are needed to clarify this important point, it is tempting to speculate that modulating the interaction between TRAIL and CRT might be used to enhance the induction of a tumour specific immune response following treatment with TRAIL. In this respect

it is worth mentioning a recently reported cell penetrating peptide that induces a marked translocation of CRT to the cell surface [22]. In addition, recombinant CRT can be used to adsorb to apoptotic cells and increase cell surface levels of CRT [23].

In conclusion, we demonstrate here that the targeted delivery of TRAIL to either the tumour or T-cell surface can potently inhibit the growth of metastatic or disseminated cancer. These findings open up novel perspectives for the treatment of ovarian carcinoma.

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Nederlandse samenvatting

Introductie

Per jaar overlijden er wereldwijd ongeveer acht miljoen mensen aan de gevolgen van kanker. Ondanks verbeterde behandelingsmethodes wordt er verwacht dat dit aantal de komende jaren helaas nog verder zal toenemen. Er wordt daarom intensief gezocht naar nieuwe methodes om kanker te voorkomen en beter te behandelen.

In grote lijnen valt kanker onder te verdelen in solide kanker (tumoren) en bloedkanker (leukemie/lymfomen). Bij solide kanker wordt als behandeling vaak gekozen voor een radicale chirurgische verwijdering van de tumor eventueel in combinatie met chemo en/of radiotherapie. In veel gevallen slaat deze behandeling in eerste instantie goed aan. Helaas komt de ziekte vroeg of laat toch weer terug. Een in het oogspringend voorbeeld hiervan is eierstokkanker (ovarium carcinoom, hierna afgekort met OC). Patiënten met OC reageren in eerste instantie goed op een dergelijke behandeling en de ziekte lijkt te zijn overwonnen. Echter, na deze periode van zogeheten “minimal residual disease” komt de kanker vaak weer terug. Helaas is dat stadium de kanker vaak uitgezaaid en resistent geworden voor verdere therapie. Er wordt gedacht dit het gevolg is van therapieresistente kankercellen die de eerste ronde van therapie om de een of andere reden hebben overleefd. Deze kankercellen groeien vervolgens weer uit. Het is dan ook noodzakelijk om nieuwe complementaire therapieën te ontwikkelen om ook deze therapieresistente kankercellen te kunnen elimineren. Een therapeutische aanpak die in dit opzicht veelbelovend lijkt is het gebruik van cellen of moleculen van het immuunsysteem.

Het immuunsysteem

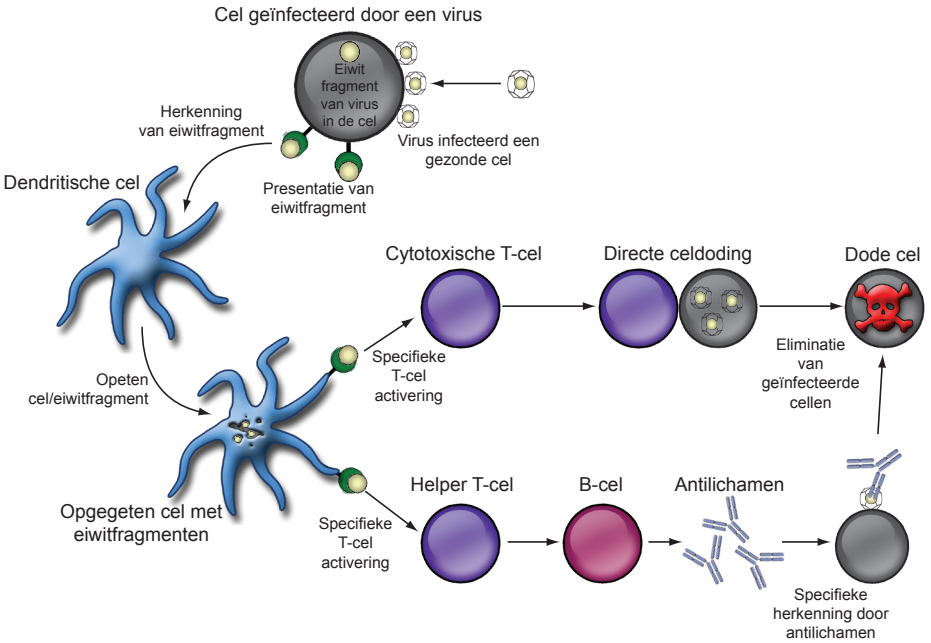
Het immuunsysteem is het verdedigingsmechanisme van het lichaam tegen aanvallen (pathogenen) van buiten af, maar ook van binnen uit. Het immuunsysteem beschermt bijvoorbeeld tegen virussen of bacteriën die het lichaam willen binnendringen. De verdediging door het immuunsysteem valt in principe te splitsen in het aangeboren en het verworven immuunsysteem.

Onder het aangeboren immuunsysteem vallen natuurlijke barrières zoals huid en slijmlagen, maar ook cellen zoals macrofagen en dendritische cellen (DCs). Het aangeboren immuunsysteem herkent algemene kenmerken van pathogene micro-organismen en vormt een eerste verdedigingslinie. Een binnengedrongen pathogene organisme wordt herkend en opgegeten door macrofagen/DCs (phagocytose). De macrofaag/DC doodt het pathogene micro-organisme, breekt het vervolgens af en zorgt voor de activatie van andere immuuncellen (o.a. cellen van het verworven immuunsysteem).

Het verworven immuunsysteem bestaat voornamelijk uit T-cellen en B-cellen die ontstaan uit voorlopercellen in het beenmerg. T voorloper cellen migreren vervolgens naar de Thymus en ontwikkelen zich hier vervolgens verder tot volwassen T-cellen. Het rijpen van B-cellen vindt plaats in het beenmerg. Zowel T-cellen alsmede B-cellen zijn essentieel

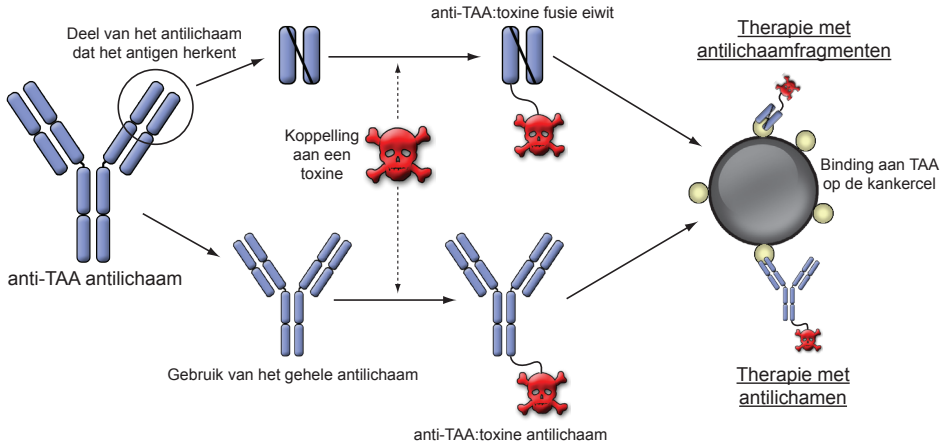
voor het tot stand komen van een adequate immuunrespons die specifiek gericht is tegen het herkende pathogene micro-organisme, maar doen dit op verschillende manier (zie voorbeeld hieronder). Zoals de naam al impliceert komt het verworven immuunsysteem tot stand door contact met allerlei antigenen. In vrijwel alle gevallen wordt het verworven immuunsysteem in eerste instantie geactiveerd door cellen van het aangeboren immuunsysteem.
Bijvoorbeeld: een cel die door een virus geïnfecteerd is breekt een deel van de virale eiwitten af en presenteert vervolgens kleine eiwitfragmenten hiervan aan DCs. DCs phagocyteren deze eiwitfragmenten en presenteren deze weer aan T-cellen en B-cellen. T-cellen herkennen vervolgens de geïnfecteerde cellen, omdat deze dezelfde eiwitfragmenten presenteren. Een deel van de T-cellen wordt nu zogeheten cytotoxische T-cellen. Na specifieke herkenning vernietigen cytotoxische T-cellen de geïnfecteerde cellen door direct cellulair contact. Een ander deel van de T-cellen (helper T-cellen) helpt (activeert) B-cellen om hun functie uit te voeren. Geactiveerde B-cellen ruimen niet direct de geïnfecteerde cellen op, maar produceren specifieke antilichamen die het betreffende antigen herkenen. Na binding aan een antigen fungeert een antilichaam als een herkenningsvlag voor andere cellen van het immuunsysteem (zie ook Figuur 1).

Het immuunsysteem en kanker
In het begin van de twintigste eeuw kwam Paul Erlich met de hypothese dat het immuunsysteem wellicht ook een belangrijke rol speelt bij het onderdrukken van kanker. Dit idee werd opnieuw opgepakt in de jaren 50 door Burnet en Thomas. Deze heren formuleerden toen de zogenaamde “immuun surveillance” theorie voor kanker. Volgens deze theorie ontstaan er voortdurend nieuwe kankercellen in het lichaam die worden herkend en opgeruimd door cellen van het immuunsysteem. Hierdoor zou het ontstaan van kanker in de meeste gevallen vroegtijdig onderdrukt worden. Sindsdien zijn er een groot aantal wetenschappelijke publicaties geweest die deze theorie bevestigen en “immuun surveillance” aanmerken als een belangrijk beschermingsmechanisme tegen het ontstaan van tumoren.
Bij “immuun surveillance” wordt er gedacht dat het immuunsysteem reageert op antigenen die selectiever tot expressie komen op tumorcellen dan op normale cellen. Deze antigenen worden daarom ook wel tumor geassocieerde antigenen (TAAs) Genoemd. Zoals hierboven vermeld kunnen antigenen herkend worden door de bijbehorende antilichamen. Antilichamen die binden aan TAAs zullen dus preferentieel binden aan kankercellen. Deze eigenschap van TAA antilichamen is dan ook gebruikt om dergelijke antilichamen verder te ontwikkelen in kankertherapieën. Een voorbeeld hiervan is het gebruik van TAA antilichamen om selectief toxische lading (bijvoorbeeld bacteriële toxinen) af te leveren aan kankercellen. Dit kan door antilichamen of antilichaamfragmenten chemisch of genetisch te koppellen aan een toxische lading (Figuur 2). Echter, degelijke toxinen zijn meestal ook



Figuur 1. Een voorbeeld van een immuunreactie. Een lichaamscel die geïnfecteerd is door bijvoorbeeld een virus breekt een deel van de virale eiwitten af en presenteert deze als eiwitfragmenten op het oppervlak van de cel. Een dendritische cel (DC) herkent deze fragmenten en phagocyteert een celfragment of de gehele cel. De DC verwerkt deze eiwitfragmenten verder en presenteert deze aan T-cellen. T-cellen worden hierdoor geactiveerd en kunnen hierna specifiek de eiwitfragmenten herkennen op het oppervlak van geïnfecteerde cellen. Cytotoxische T-cellen kunnen hierna direct geïnfecteerde cellen doden, terwijl helper T-cellen de B-cellen activeren. B-cellen produceren vervolgens antilichamen die specifiek de eiwitfragmenten (antigenen) herkennen. Deze herkenning maakt de geïnfecteerde cellen als het ware zichtbaar voor de rest van het immuunsysteem. Dit resulteert uiteindelijk ook in de dood van de geïnfecteerde cel.

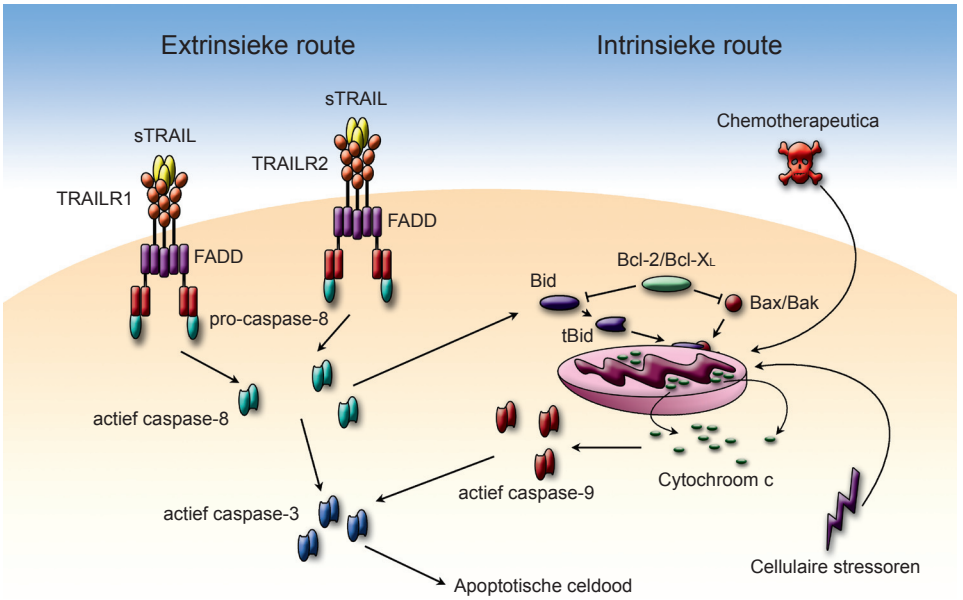
giftig voor gezonde cellen. Omdat TAAs ook kunnen voorkomen op een gedeelte van de gezonde cellen zullen deze ook getroffen worden door het toxine. Een ideale lading voor antilichamen zou dan ook 1. alleen toxisch moeten zijn voor tumorcellen en 2. pas actief worden als het bindt aan tumorcellen. Een molecuul dat hier uitermate geschikt voor lijkt te zijn is “Tumor Necrosis Factor (TNF) Related Apoptosis Inducing Ligand” (TRAIL).
TRAIL is een zogeheten type II transmembraan eiwit dat voorkomt op het celoppervlak van bepaalde immuuncellen. TRAIL wordt door deze immuuncellen gebruikt om kankercellen te doden via een proces dat geprogrammeerde celdood (apoptose) heet. Apoptose wordt door het lichaam gebruikt om overvloedige of mogelijk gevaarlijk geworden snel en veilig op te ruimen. Een belangrijke eigenschap van TRAIL voor kankertherapie is dat TRAIL alleen apoptose lijkt te induceren in cellen die kwaadaardig zijn en normale cellen volledig



Figuur 2. Het maken van antikanker therapeutica gebaseerd op antilichamen. Een antilichaam herkent specifiek een bepaald tumor geassocieerd antigen (TAA). Deze herkenning vindt plaats via de zogeheten complementair herkennende regionen (CDRs; zie omlijning). Therapeutica kunnen dan ook gemaakt worden door het hele antilichaam te gebruiken (onderste lijn) , alleen de CDRs (bovenste lijn), of variaties hiervan. Antilichamen of antilichaamfragmenten kunnen chemisch of genetisch gekoppeld worden aan een toxine. Hierdoor ontstaat een anti-TAA:toxine fusie-eiwit dat specifiek een TAA op een kankercel kan herkennen. Hierdoor kunnen toxinen aan het oppervlak van kankercellen worden afgeleverd. Na aflevering worden de toxinen meestal opgenomen in de cel. Dit resulteert uiteindelijk dan in de dood van de kankercel.

ontziet.

Apoptose kan worden geïnduceerd door de zogeheten intrinsieke en extrinsieke routes (Figuur 3). De intrinsieke route wordt geactiveerd van binnen uit de cel, terwijl de extrinsieke route wordt geactiveerd door receptoren op de buitenkant van de cel. Dit laatste is het geval bij TRAIL. TRAIL induceert apoptose door te binden aan TRAIL-receptoren (TRAILRs) op het oppervlak van de cel. Er zijn vijf verschillende TRAILRs te onderscheiden en deze dragen verschillend bij aan de inductie van apoptose. TRAILR1 en TRAILR2 induceren apoptose in cellen, terwijl er van TRAILR3 en TRAILR4 gedacht wordt dat deze fungeren als een soort van afleidingsreceptor. TRAIL kan wel aan TRAILR3 en TRAILR4 binden, maar dit resulteert niet in apoptose (Figuur 4). Een andere variante TRAIL receptor, OPG, komt niet op cellen voor, maar is oplosbaar. Ook TRAIL kan in oplosbare vorm voorkomen (sTRAIL) in het lichaam. Dit sTRAIL is nagemaakt in laboratoria en heeft ook een antikanker werking. Echter, van sTRAIL is bekend dat dit veel minder goed in staat is om TRAILR2 te activeren. Hiermee lijkt sTRAIL dus ook grotendeels te voldoen aan de criteria voor een ideale lading voor aflevering aan kankercellen via antilichaamfragmenten. Namelijk: sTRAIL is niet toxisch voor gezonde cellen en induceert voornamelijk apoptose als het aan het oppervlak van kankercellen gebonden is.

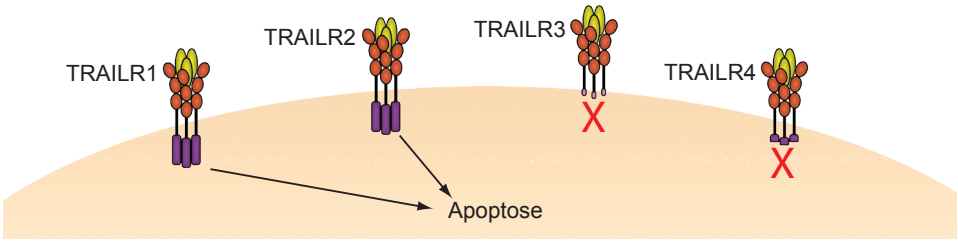


Figuur 3. Inductie van apoptose. Geprogrammeerde celdood ofwel apoptose vindt plaats door een gecontroleerde afbraak van de cel van binnen uit. Deze afbraak is afhankelijk van zogeheten cellulaire proteasen genaamd caspases. Deze caspases knippen als het ware een cel van binnenuit op een gecontroleerde manier kapot. Dit resulteert uiteindelijk in de dood van de cel. Apoptose kan worden aangezet (geïnduceerd) via de zogeheten extrinsieke en intrinsieke routes. Bij de extrinsieke route bindt een eiwit zoals sTRAIL aan TRAIL receptoren (TRAILR) die zich op het oppervlak van de cel bevinden. Hierna ontstaat een ketting reactie die zorgt voor het activeren van de caspases en uiteindelijk apoptotische celdood. Bij de intrinsieke route kunnen chemotherapeutica, toxinen, of andere cellulaire stressoren zorgen voor beschadiging van de mitochondriën. De mitochondriën zijn als het ware de energiefabrieken van de cel en als deze beschadigd raken wordt er een proces in gang gezet om de cel op een gecontroleerde manier via caspases een apoptotische celdood te laten ondergaan. sTRAIL=soluble TRAIL, TRAILR=TRAIL receptor, FADD=Fas associated death domain, Bid=BH3 interaction death domain agonist, tBid= truncated Bid, Bcl-2= B-cell lymphoma 2, Bcl-XL= B-cell lymphoma XL, Bax=Bcl-2 associated X protein, Bak=Bcl-2 homologous antagonist/killer.

Dit in ogenschouw nemend hebben wij voorheen dan ook fusie eiwitten gemaakt waarin sTRAIL genetisch gekoppeld is aan antilichaamfragmenten die binden aan TAAs op het oppervlak van kankercellen (Figuur 5). In de volgende paragrafen wordt het onderzoek beschreven naar het gebruik van dergelijke fusie eiwitten bij het selectief en veilig doden van kankercellen.

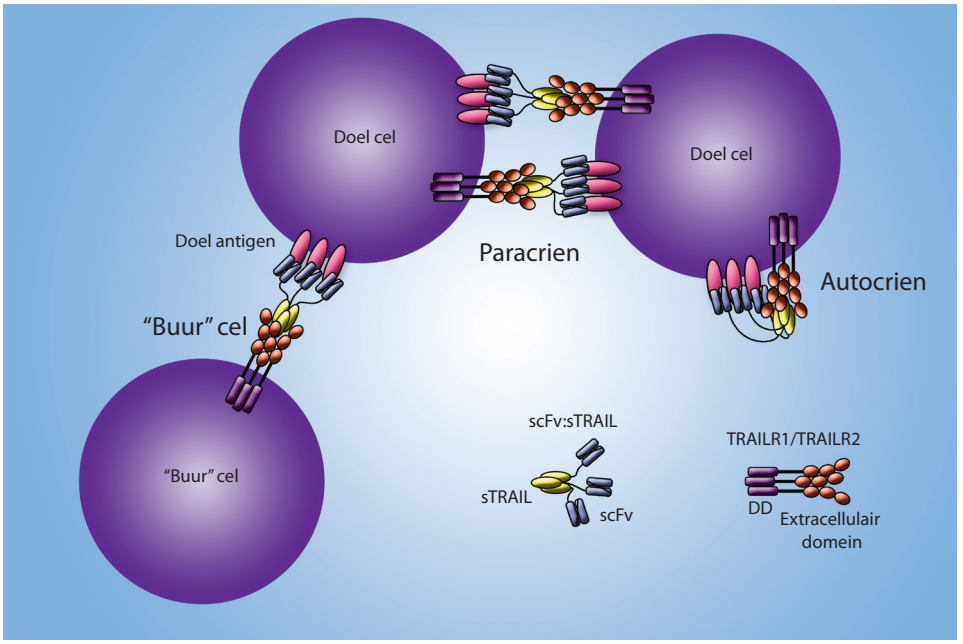
Het selectief afleveren van TRAIL aan het oppervlak van kankercellen

Hoofdstuk 2 geeft een korte samenvatting van de publicaties met sTRAIL fusie eiwitten tot



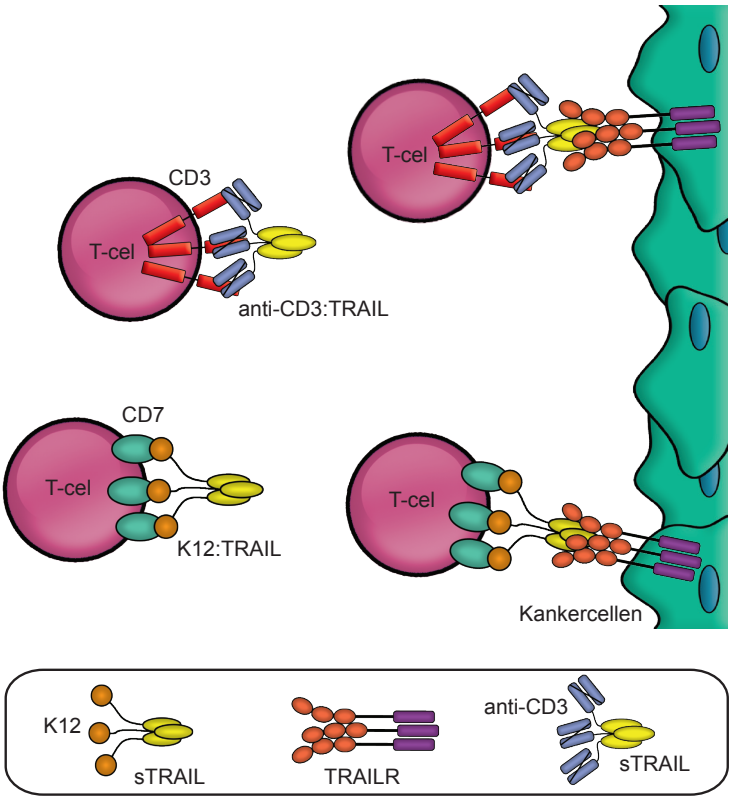
Figuur 4. TRAIL receptoren. TRAIL kan binden aan vier verschillende TRAIL receptoren. TRAILR1 en TRAILR2 hebben een intracellulair domein waaraan FADD kan binden en apoptose kan worden geïnduceerd (zie ook Figuur 3). TRAILR3 en TRAILR4 hebben geen intracellulair domein waar FADD aan kan binden en kunnen dus ook geen apoptose induceren.

nu toe. Hierbij ligt de nadruk op het toepassing van het zogenaamde “dual signaling” concept. Hierbij wordt er in een sTRAIL fusie-eiwit gebruik gemaakt van een antilichaamfragment dat van zichzelf ook een antikankerwerking heeft. Een belangrijk voorbeeld hiervan is het fusie eiwit scFv425:sTRAIL. In dit fusie-eiwit is het antilichaam fragment scFv425 gekoppeld aan sTRAIL. ScFv425 bindt aan de Epidermale Groei Factor Receptor (EGFR) welke tot over-expressie komt op het oppervlak van tumorcellen van vele soorten solide tumoren. EGFR is de receptor voor de Epidermale Groei Factor (EGF), waarbij binding van EGF aan EGFR zorgt voor versnelde tumorgroei. Doordat scFv425 deze binding verhindert, groeien de tumorcellen minder snel en worden ze tevens gevoeliger voor sTRAIL. Voorheen hebben wij laten zien dat scFv425:sTRAIL in staat is om in vitro (in cellen in het laboratorium) selectief EGFR-positieve kankercellen van verschillende oorsprong te doden. In **hoofdstuk 3** hebben wij deze veelbelovende resultaten verder uitgebouwd door de antitumoractiviteit van scFv425:sTRAIL te bepalen in vivo (in proefdieren). In deze proefdieren hebben wij gekozen voor een zogeheten intraperitoneaal (buikholte) tumormodel. Dit tumormodel bootst de situatie na van patiënten met uitgezaaide kanker in de buikholte (zoals bij bijvoorbeeld OC en darmkanker). Om een adequate concentratie van scFv425:sTRAIL in de muis te garanderen hebben wij gebruik gemaakt van een recombinant adenovirus dat codeert voor dit fusie-eiwit (Ad-scFv425:sTRAIL). Na infectie van een proefdier infecteert Ad-scFv425:sTRAIL voornamelijk de lever van de muis, waarna de lever als het ware gaat fungeren als een fabriek voor de productie van scFv425:sTRAIL. Hierdoor wordt er een aanzienlijke en constante hoeveelheid scFv425:sTRAIL door de muis zelf geproduceerd en komt scFv425:sTRAIL terecht in de bloedbaan. ScFv425:sTRAIL kan vervolgens binden aan de tumorcellen in de buikholte van de muis en induceert selectief apoptose in deze kankercellen. Logischerwijs is de concentratie scFv425:sTRAIL in de bloedbaan afhankelijk van de hoeveelheid Ad-scFv425:sTRAIL (virus titer) die in eerste instantie wordt ingespoten bij de muis. Injectie van muizen met een grote hoeveelheid Ad-scFv425:sTRAIL (hoge



Figuur 5. Het selectief afleveren van TRAIL aan het oppervlakte van kankercellen. Zoals ook in Figuur 2 is beschreven kan een toxische lading worden afgeleverd aan het oppervlak van kankercellen met behulp van een antilichaamfragment. Ditzelfde is mogelijk voor het selectief afleveren van TRAIL. In onze strategie is TRAIL genetisch gekoppeld aan een zogeheten scFv antilichaamfragment. Omdat TRAIL trimeer voorkomt (drie TRAIL moleculen vormen automatisch een trimeer molecuul) zullen er daarom ook drie scFv fragmenten binnen een TRAIL fusie-eiwit voorkomen. Een dergelijk scFv:sTRAIL fusie-eiwit kan vervolgens binden aan een doel antigen op het oppervlak van kankercellen. Hiermee wordt het sTRAIL omgezet naar een membraan vorm van TRAIL en induceert het apoptose in de kankercellen. Dit kan plaatsvinden op een autocriene manier (binden en doden van dezelfde cel), maar ook paracrien (het over en weer doden van buurcellen). Een bijkomend voordeel van een dergelijk fusie-eiwit is dat het tevens apoptose induceert in "buur" kankercellen die dit antigen niet hebben, maar wel gevoelig zijn voor TRAIL.

titer) resulteerde dan ook in hoge niveaus van scFv425:sTRAIL in de bloedbaan, hetgeen leidde tot volledig elimineren van de aanwezige tumoren in de buikholte. Muizen die geïnjecteerd werden met een kleinere hoeveelheid Ad-scFv425:sTRAIL (lage titer) hadden inderdaad minder scFv425:sTRAIL in het bloed (<10 ng/ml), maar dit bleek wel voldoende om de groei van de tumoren volledig te remmen. Tezamen geven deze resultaten aan dat scFv425:sTRAIL veelbelovend is voor de behandeling van uitgezaaide kanker in de buikholte. Toepassing van deze benadering bij mensen ligt echter niet voor de hand vanwege allerlei praktische en principiële bezwaren van gentherapie met adenovirussen.
Vervolgens hebben wij gekeken of we inductie van apoptose door middel van scFv425:sTRAIL



Figuur 6. Het bewapenen van T-cellen met behulp van TRAIL. Zoals ook in Figuur 5 is beschreven kan TRAIL selectief worden afgeleverd aan het oppervlak van cellen met behulp van een antilichaamfragment. Ditzelfde is mogelijk voor het afleveren van TRAIL aan het oppervlak van T-cellen. Als variatie hierop hebben wij echter in hoofdstuk 6 ook gebruik gemaakt van een receptor-ligand in plaats van een scFv antilichaamfragment. Het principe is hierbij hetzelfde, maar er wordt gebruik gemaakt van een natuurlijke bindingspartner van een receptor op het oppervlak van T-cellen. In dit geval het K12-CD7 ligand-receptor paar. Op het moment dat deze fusie-eiwitten anti-CD3:TRAIL of K12:TRAIL binden aan het oppervlak van de T-cel wordt de T-cel als het ware "bewapend" met een extra kankerdodend eiwit (TRAIL). Dit TRAIL gebruikt de T-cel vervolgens om de kankercellen te doden.

nog verder konden versterken door de binding van TRAIL aan de verschillende TRAIL receptoren te moduleren. Zoals vermeld zijn er vijf verschillende TRAIL receptoren, waarvan er vier op het oppervlak van cellen voorkomen en één een oplosbare receptor is. Van de vier celgebonden TRAIL receptoren induceren alleen receptor 1 (TRAILR1) en receptor 2 (TRAILR2) apoptose. TRAILR3 en TRAILR4 binden wel aan TRAIL, maar induceren geen apoptose. Dit in ogenschouw nemende hebben verschillende onderzoeksgroepen varianten van sTRAIL gemaakt die meer binden aan TRAILR1 of TRAILR2 en minder aan TRAILR3 en TRAILR4. Van één van deze varianten (sTRAILmR1-5) is gepubliceerd dat deze voornamelijk bindt aan TRAILR1. Daarom was onze gedachtegang dat deze variant wellicht meer apoptose kan induceren in cellen die meer TRAILR1 tot expressie brengen vergeleken met TRAILR2. Om dit te onderzoeken hebben wij in **hoofdstuk 4** een nieuw fusie-eiwit geproduceerd genaamd scFv425:sTRAILmR1-5. Dit fusie-eiwit bindt net als

scFv425:sTRAIL aan EGFR en induceert vervolgens selectief apoptose in tumorcellen die EGFR tot expressie brengen. Interessant genoeg induceerde scFv425:sTRAILmR1-5 zeer efficiënt apoptose in tumorcellen, ongeacht de expressie niveaus van de verschillende TRAIL receptoren. Hiernaast was de apoptotische activiteit van scFv425:sTRAILmR1-5 ook sterker dan scFv425:sTRAIL wanneer deze gecombineerd werden met chemotherapeutische behandelingen. Tezamen geeft dit aan dat het gebruik van sTRAIL varianten in het fusie-eiwit concept kan leiden tot verhoogde antikanker activiteit.

Kankerpatienten waarbij de kanker in een vroeg stadium wordt vastgesteld hebben meestal een goede kans op genezing. Hiertegenover staat dat patiënten met uitgezaaide kanker uiteindelijk meestal overlijden aan de gevolgen van deze metastases. Een belangrijke factor hierbij zijn therapieresistente kankercellen die de eerste behandelingen overleven en vervolgens weer uitgroeien tot tumoren/metastases. Het is voor een genezende behandeling van kanker dan ook van groot belang om te voorkomen dat tumoren metastases gaan vormen. Onze hypothese was dat het “dual signaling” concept van bepaalde fusie-eiwitten gebruikt zou kunnen worden om selectief de vorming van metastases te remmen en tegelijkertijd apoptose te induceren via TRAIL. Hiertoe zou gebruik gemaakt kunnen worden van een scFv fragment dat van zichzelf een antimetastaserende werking heeft. In **hoofdstuk 5** hebben wij deze hypothese getest in het kader van melanoom, een kanker die gekenmerkt wordt door de snelle en uitgebreide vorming van metastases. Hiertoe hebben wij een nieuw fusie-eiwit ontwikkeld genaamd anti-MCSP:TRAIL. Het antilichaamfragment in anti-MCSP:TRAIL is afkomstig uit een antilichaam dat bindt aan melanoomcellen, hetgeen een antimetastaserende werking blijkt te hebben. MCSP staat voor Melanoom Chondroitin Sulfate Proteoglycan en komt verhoogd tot expressie op melanoomcellen. Overexpressie van MCSP lijkt de vorming van metastases door melanoomcellen te bevorderen. Behandeling met Anti-MCSP:TRAIL bleek in staat om apoptose te induceren in een serie verschillende melanoomcellen en kon de groei remmen van melanoomcellen zowel in vitro als in vivo. Een interessant observatie die wij maakten was dat behandeling met Anti-MCSP:TRAIL zelfs de groei remde van TRAIL-resistente melanoom cellen. Daarom speculeerden wij dat het groeiremmende effecten van anti-MCSP:TRAIL wellicht een gevolg was van de remming van MCSP. Na verder onderzoek bleek inderdaad dat anti-MCSP:TRAIL een aantal signaleringsroutes remde die belangrijk zijn voor metastasering van melanoomcellen. Hieruit concluderen wij dat anti-MCSP:TRAIL veelbelovende antimelanoom activiteit heeft die berust op de gelijktijdige remming van signalering die metastases bevordert en de inductie van apoptose.

Het selectief afleveren van TRAIL aan het oppervlak van immuuncellen

Hierboven is beschreven hoe het selectief afleveren van TRAIL aan het oppervlak van kankercellen gebruikt kan worden om kankercellen selectief te elimineren. Hierbij hebben

wij vastgesteld dat het scFv:TRAIL concept: 1. in vivo antikanker activiteit voor tumoren in de buikholte, 2. versterkt kan worden door gebruik te maken van bepaalde TRAIL varianten, 3. de groei van melanomen kan remmen en 4. geen of nauwelijks toxische effecten heeft ten opzichte van gezonde normale cellen.
Vervolgens hebben wij dit concept van het selectief afleveren van TRAIL aangepast door TRAIL niet aan kankercellen, maar juist aan immuuncellen af te leveren. Het idee hierachter is dat hierdoor de antikankerwerking van deze immuuncellen versterkt zal worden. Zoals beschreven staat in hoofdstuk 2 van dit proefschrift komt TRAIL van nature voor op het celoppervlak van zogenaamde NK-cellen, NK-cellen zijn onderdeel van het aangeboren immuunsysteem en vervullen een rol die sterk complementair is aan de rol van T-cellen. Namelijk, kwaadaardige cellen die MHC-klasse moleculen verlaagd, of geheel niet meer tot expressie te brengen, kunnen ontsnappen aan vernietiging door T-cellen. NK-cellen herkennen en vernietigen echter cellen die geen of te weinig MHC-I tot expressie brengen. TRAIL speelt hierbij belangrijk rol. Het TRAIL op NK-cellen is dus ook belangrijk voor het vernietigen van b.v. metastaserende kankercellen. T-cellen brengen normaal veel minder TRAIL tot expressie op het oppervlak dan NK-cellen. Echter, onder bepaalde omstandigheden kunnen T-cellen wel gestimuleerd worden om meer TRAIL op het oppervlak tot expressie te brengen en dit versterkt de antikankerwerking vervolgens enorm. Tezamen geeft dit aan dat het kunstmatig verhogen van de hoeveelheid TRAIL op het oppervlak van T-cellen de antikankerwerking van deze T-cellen aanzienlijk zou kunnen versterken. Dit zou kunnen worden bereikt door TRAIL selectief af te leveren aan het oppervlak van immuuncellen met behulp van scFv antilichaamfragmenten. Deze hypothese hebben wij in **hoofdstuk 6** getest door TRAIL selectief af te leveren aan de T-cel oppervlaktemoleculen CD3 en CD7, met behulp van respectievelijk de fusie-eiwitten anti-CD3:TRAIL en K12:TRAIL (Figuur 6). Beide fusie-eiwitten waren in staat om de antikankeractiviteit van T-cellen aanzienlijk (> 500-voudig) te versterken t.o.v. een grote verscheidenheid aan kankercellijnen. Daarnaast werd ook de antikankeractiviteit van T-cellen versterkt t.o.v. kankercellen die uit patiëntentumoren geïsoleerd waren. Hierop volgend hebben wij de activiteit van deze fusie-eiwitten getest in muizen met een tumor in de buikholte. Hieruit bleek dat beide fusie eiwitten in staat waren om de groei van dergelijke tumoren aanzienlijk te remmen. Tezamen geeft dit aan dat selectieve aflevering van TRAIL aan het oppervlak van T-cellen de antikanker activiteit van deze cellen aanzienlijk kan versterken. Een dergelijke strategie zou relatief simpel toegepast kunnen worden in reeds bestaande immuuntherapieën voor kanker.

TRAIL en tumor immuniteit

Zoals hierboven beschreven kan TRAIL gebruikt worden om de antikankeractiviteit van immuuncellen te versterken. Ook zijn er aanwijzingen dat TRAIL nog andere immuunstimul-

erende effecten zou kunnen hebben. Onlangs is gepubliceerd dat, voorafgaand aan apoptose in kankercellen, het intracellulaire eiwit calreticuline (CRT) zich naar het oppervlak van de cel verplaatst. Dit gebeurt ten gevolge van behandeling met bepaalde apoptose inducerende therapeutica, waaronder TRAIL. Eenmaal op het celoppervlak dient CRT als een herkenningsmolecuul voor DCs en bevordert de opname van apoptotische kankercellen. Opname door DCs is een belangrijke vereiste voor het ontstaan van een immuunreactie die specifiek is gericht tegen deze kanker. Opname van apoptotische kankercellen door andere immuuncellen, zoals macrofagen, leidt juist niet tot een immuunreactie en kan leiden tot de inductie van tolerantie. CRT is dus een belangrijke schakel om te bepalen of apoptotische cellen wel of geen immuunreactie induceren. Tevens kwam er onlangs ook bewijs naar voren dat TRAIL direct kon binden aan CRT. Daarom hebben wij in **hoofdstuk 7** gekeken naar de mogelijke rol van CRT bij de apoptose zoals die geïnduceerd wordt na behandeling met TRAIL. Wij ontdekten dat behandeling van kankercellen met TRAIL resulteerde in een snelle pre-apoptotische associatie van CRT met TRAIL en TRAILR2. Tegelijkertijd liet CRT lost van zijn normale bindingspartner CD47. Van CD47 wordt gedacht dat het verhindert dat CRT bindt aan phagocyten. Wanneer op de celmembraan CRT vrijkomt van CD47, dan verhoogt dat de phagocytose van de betreffende cel door fagocyten. Verder ontdekten wij dat de binding van CRT aan TRAIL geen invloed had op de inductie van apoptose door TRAIL. Concluderend, behandeling van kankercellen met TRAIL heeft direct invloed op CRT aan het celoppervlak van kankercellen. Deze bevinding kan wellicht verder implicaties hebben voor het ontwikkelen van een antikanker immuunreactie.

Conclusies

In dit proefschrift hebben wij laten zien dat het selectief afleveren van TRAIL, of rationeel ontworpen varianten van TRAIL, aan het oppervlak van kankercellen deze kankercellen kan doden. Ook hebben wij laten zien dat de antikanker activiteit van T-cellen versterkt kan worden door het selectief afleveren van TRAIL aan het oppervlak van deze cellen. Onze data toont verder aan dat scFv:TRAIL fusie eiwitten, die tevens gebruik maken van het zogenaamde ‘dual signalling concept’, de groei van kankercellen op meerdere manieren kunnen remmen en dat dit resulteert in een versterkte antikanker werking. De behaalde resultaten bieden dus perspectief voor het toepassen van scFv:TRAIL fusie eiwitten bij de behandeling van kanker.

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Dennis Jager, Manon van Riezen, Marleen Rinket, Marleen van Oosten en Harm-Jan Lou-

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Marco